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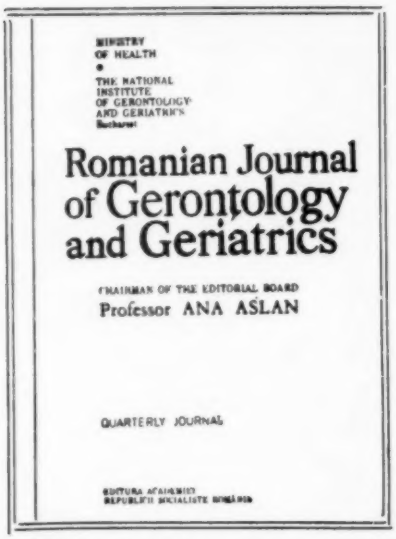
Romanian Journal of Gerontology and Geriatrics

Editor: Professor Doctor Ana Aslan, member of the Academy of the Socialist Republic Romania, General Director of the National Institute of Gerontology and Geriatrics, Bucharest, Romania.



The major goal of the Romanian Journal of Gerontology and Geriatrics is to promote clinical, psychological, social and basic research in the field of gerontology and geriatrics. It pays special attention to the prophylaxis and treatment of premature aging and age-related chronic diseases, as well as to problems of organization and guidance of medical and social care for the aged. The journal has also promoted the interdisciplinary collaboration of specialists working in various fields: clinical, biology, biochemistry, biophysics, genetics, immunology, pharmacology, psychology, endocrinology, neurology, demography, sociology, as well as of all specialists interested in the problems of the third age. In the aim to establish an exchange of scientific information with foreign countries, the journal is published in several languages and is open for participation, also to foreign specialists, belonging to various fields.

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EDITORIAL

THIS VOLUME of *Experimental Gerontology* marks the beginning of the 20th year of its publication. The journal was founded in 1965 by Dr. Alex Comfort, who attended its birth, guided it through its childhood, and ministered to its needs until now, its 20th birthday. Alex's tenure as Editor-in-Chief is not only remarkable for its longevity, but also for the high standards that he established for this journal.

Twenty years ago, research on the biology of aging, or biogerontology, was a backwater of scientific investigation. Few senior people worked in the field and even fewer young scientists chose it as their profession. One of those senior people was Alex Comfort. Not only did Alex make significant scientific contributions to experimental gerontology itself, but he had the vision to promote interest in what he believed was an important discipline that deserved greater attention by the scientific community.

Alex achieved this goal in at least two ways. In 1956, he published his book *The Biology of Senescence,* which has since appeared in a second edition in 1964 and in a third edition in 1978. That book had a profound impact on the careers of many young biologists who chose biogerontology as their vocation because of the excitement this book stimulated. The second way in which Alex promoted biogerontology was to found *Experimental Gerontology*. Few journals were dedicated to publishing research on the biology of aging in 1965, and Alex saw *Experimental Gerontology* as filling that void, drawing attention to the field, and becoming a vehicle for the publication of high quality research reports.

Today, biogerontology is no longer a backwater. The field is enjoying a renaissance of interest that few of us thought possible 20 years ago. The founding of this journal by Alex Comfort and the quality of the papers published in it for the past 20 years have contributed enormously to the current activity and excitement in this field. We are all richer for Alex's foresight and determination.

Now Alex has decided to relinquish his post as Editor of *Experimental Gerontology* to coincide with his return to England from California. We wish him well and are grateful to him for agreeing to remain as an Associate Editor, where his advice and counsel still will be available.

Experimental Gerontology now has been restructured significantly. A new group of 27 Associate Editors has been appointed. This represents a significant increase in the number of previous editorial board members. This change should result in a faster review process and the likelihood that most of the disciplines that presently compose the broad area of biogerontology will be represented by our new board of Associate Editors.

What will not change is the aim of *Experimental Gerontology*. That aim will continue to be the publication of research results that elucidate the processes of biological aging in all living things from the molecular level to that of the whole organism. However, the

scope of *Experimental Gerontology* will be expanded to include not only reports of original biological research, but review articles, theoretical or speculative articles, actuarial and mathematical articles, editorials, book reviews, meeting reports, announcements and news items, letters to the editor, and occasional special articles.

There will also be a number of other changes in *Experimental Gerontology*. Some will be subtle and some not so subtle. Most will involve style, layout, and type face. We are also going to expand significantly the section on instructions to authors. We hope that this will clarify ambiguities, rather than burden authors with additional requirements.

Experimental Gerontology will continue to be a bi-monthly, peer-reviewed journal with international coverage. High scientific standards will govern the choice of accepted papers. Meritorious typescripts are encouraged for submission to the Editor at the address given elsewhere in this issue. We also solicit editorials, letters to the editor, announcements and news items. Those who plan to submit review articles should first discuss their intentions with the Editor.

We hope that you will be pleased with the new *Experimental Gerontology* and that you will consider publishing your results in aging research in a journal that, appropriately, is comparatively old and justly venerable.

Leonard Hayflick
Editor-in-Chief

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RAPID IN VIVO QUANTIFICATION AND COMPARISON OF HYDROPEROXIDES AND OXIDIZED COLLAGEN IN AGING MICE, RABBITS AND MAN

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Abstract—A new method is described for the measurement of hydroperoxides and oxidized collagen in epidermal skin of mice, rabbits and man using reflective, near-infrared spectroscopy. Slight decreases in levels of hydroperoxides and oxidized collagen occur during the non-senescent phase of life. On the other hand, dramatic increases are experienced during the senescent phase. These increases are smallest in man, who has the most efficient defence system against peroxides and other active oxygens. Preliminary clinical work suggests that significantly reduced levels of hydroperoxides and oxidized collagen can be obtained with antioxidant diet-supplementation in middle-aged mice (~90%, ~34%) and man (~19%), respectively.

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INTRODUCTION

A QUANTIFIED investigation of *in vivo* generated free radicals and subsequent artifacts of free-radical reactions in living systems has been lacking. Specifically, hydroperoxides and oxidized collagen have not been previously detected and measured directly *in vivo* (Donato Jr., 1981). This paper describes a new method for the measurement of hydroperoxides and oxidized collagen in epidermal skin of aging mice, rabbits and man using reflective, near-infrared spectroscopy.

MATERIALS AND METHODS

32 humans, 17 English rabbits and 56 Sprague-Dawley mice of varying ages and sexes were measured on their shaved epidermis with a reflective near-infrared spectrometer ("Inframatic 8100," Basab Inc., Segeltorp, Sweden). Interchangable IR interference filters at 1457 and 1586 ± 33nm were used for determination of hydroperoxide (R-OOH) and amide bond (-CONH-) levels, respectively. These two wavelengths are highly specific to quantitative changes in hydroperoxides (Holman and Edmonson, 1956), and amide bonds (Bath and Ellis, 1941).

RESULTS

Figure 1 indicates a slight decrease in hydroperoxide levels in rabbits and man, and an unchanged level in mice during the non-senescent phase of life. During the senescent phase, both man and mice experience dramatic increases in levels of hydroperoxides.

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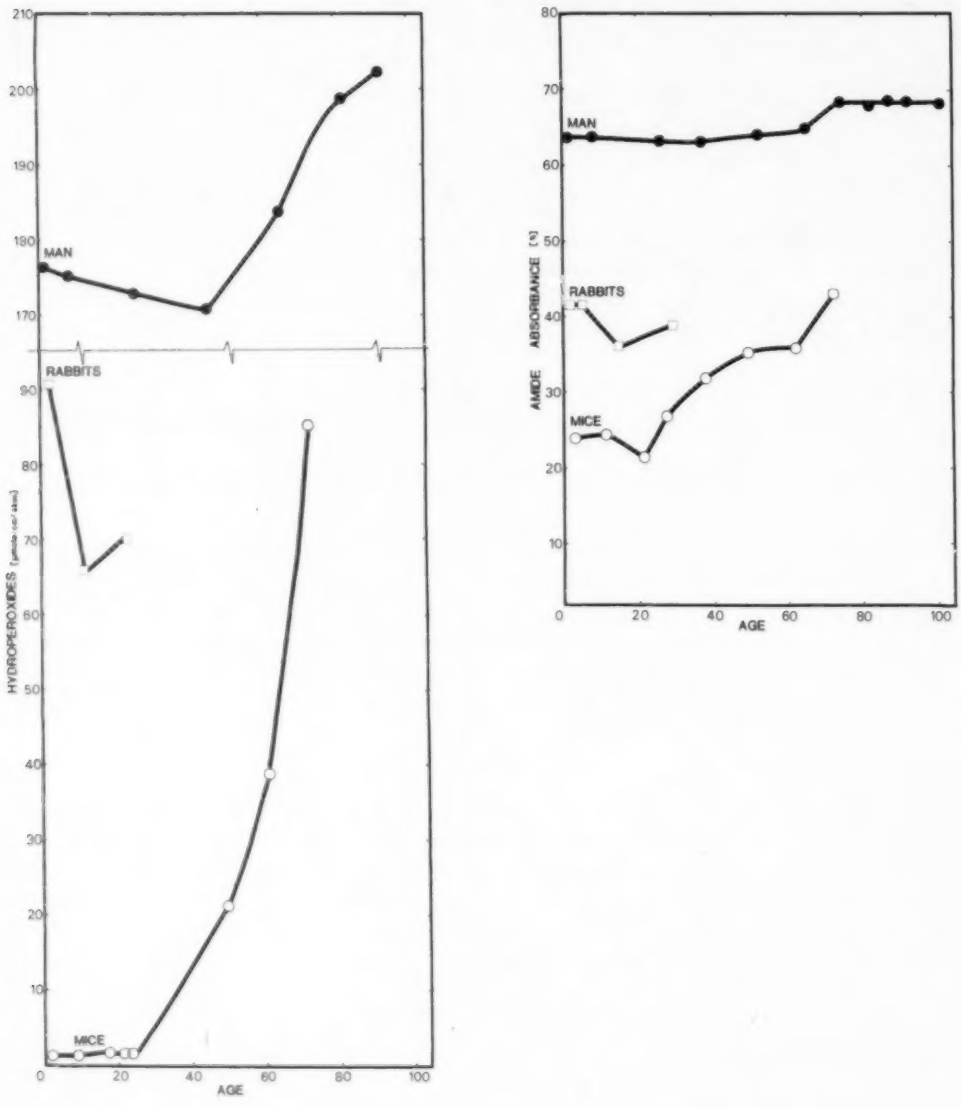

FIG. 1 & 2. A species comparison of the changes in concentration of hydroperoxides and amide bonding during the lifespans of man, rabbits and mice. For purposes of comparison, the X-axis is calibrated in years for man, 1/7.3 yr for rabbits and 1/37 yr for mice. All values were SEM ± 1.8% at a 95% level of confidence with the exception of senescent mice (± 13.9%). Calibration of hydroperoxide concentrations from absorbance percentages is presented in figure 3. Not shown are ten 8-month-old rabbits suffering cholesterol-induced arteriosclerosis which showed ~7% lower values than normal rabbits.

Preliminary results indicate that these levels in middle-aged man and mice may be lowered by ~15% and ~90%, respectively, using antioxidant food supplements (0.37% w/w, butylated hydroxytoluene). Possibly, a lowering of these levels might affect lipofuscin formation or mean lifespan (Harman, 1981).

Figure 2 indicates slight decreases in amide bonding during the non-senescent phase of life in man, rabbits and mice. During the senescent phase, mice experience dramatic increases in amide bonding while rabbits and man succumb to more gradual increases. Increases in amide bonding imply increases in collagen oxidation and subsequent collagen inelasticity during senescence (Bailey and Peach, 1971; Bailey and Shimokomaki, 1971; Robins and Bailey, 1972; Robins *et al.*, 1973; Mechanic *et al.*, 1974; Robins and Bailey, 1975; Bailey *et al.*, 1977). Similar results have been reported which demonstrate increased collagen oxidation and inelasticity in bovine skin during aging (Robins *et al.*, 1973; Bailey *et al.*, 1977). Our preliminary results indicate that these amide levels in middle-aged man and mice may be lowered ~19% and ~34%, respectively, using antioxidant food suppliments (0.37% w/w butylated hydroxytoluene and similar derivatives and mixtures).

DISCUSSION

The results of Figures 1 and 2 complement our previous work in the detection and quantification of free radicals and antioxidant mixtures. Our previous methods used site-specific, specially-synthesized chemiluminescent probes. The CML probe is site-specific to the mitochondrial inner membrane (Lippman and Ågren, 1982) and to superoxide anion radical ($O_2^{-}\bullet$) flux (Merényi and Lind, 1980). This ~6% flux is assumed to be destroyed by a mitochrondrion's first line of defence, manganese superoxide dismutase (MnSOD) (Weisiger and Fridovich, 1973). Lines of defence become increasingly more sophisticated and compartmentalized if one examines aerobes increasingly higher upon the evolutionary scale (Lippman, 1983).

$O_2^{-}\bullet$ which escapes various lines of defence is free to react in a mitochondrion with a variety of substrates. One prominent substrate is hydrogen peroxide (H_2O_2) which is known to exist in large, steady-state concentrations of millimoles per gram mitochondria in a mitochondrion's matrix pool (Nohl *et al.*, 1981). This reaction may be summarized as:

$$O_2^{-}\bullet + H_2O_2 \rightarrow \bullet OH + O_2 + {}^{-}OH$$

and it may proceed *in vitro* with or without catalysis of transition metals, for example iron (Donato, 1981). This reaction forms a slightly basic environment which may be measured directly in intact mitochondria with the CML probe to an approximate pH value of 8.1 (Lippman and Ågren, 1982). Also, this reaction forms the chemically potent hydroxyl radical (•OH) which is known to cause activation of chemical carcinogins (Walton and Packer, 1980), irreversable destruction of mitochondrial DNA in post-mitotic cells (Miquel *et al.*, 1980) and peroxidation of polyunsaturated fatty acids (PUFA) in membranes (Lippman, 1983).

Artifacts of PUFA peroxidation have been extensively investigated since the middle of the last century when lipofuscin granules were first observed by light microscopy in aging neurons (Hannover, 1842). Modern morphological methods have determined that lipofuscin is the peroxidative and enzymatic destruction of old membranes and other structures containing PUFA which occurs in the vacuome of lysosomes through a sequestering mechanism called autophagocytosis. In this regard, our group has developed

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other site-specific chemiluminescent probes which detect lysosome sensitivity to $O_2^{-}\bullet$ and other peroxidation artifacts (Lippman *et al.*, 1981).

Figures 1, 2 and 3 indicate that the hydroperoxide pool in the epidermal skin of mice contains approximately micromoles per cm^2 hydroperoxides. This order of magnitude is an estimated micromoles per gram mitochondria. This in turn may be compared with an approximate millimole per gram mitochondria of H_2O_2 in the mitochondrial matrix pool (Nohl *et al.*, 1981) and a flux of approximately 0.1 micromoles per min per gram of $O_2^{-}\bullet$ from the respiratory chain located in the mitochondrial inner membrane (Lippman and Ågren, 1982). These values may be significantly decreased by prophylactic use of antioxidants as demonstrated in rat mitochondria (Lippman, 1980), human mitochondria (Lippman, 1981) and human lysosomes (Lippman *et al.*, 1981).

Figures 1 and 2 indicate slight increases in collagen inelasticity and dramatic increases in hydroperoxides during aging. These increases are smallest in man who has the most efficient defence system against peroxides and other active oxygens (Lippman, 1983). These phenomena appear to be strongly influenced by chemically active oxygens, for example H_2O_2, $\bullet OH$ and $O_2^{-}\bullet$ and strongly inhibited by antioxidants. Reflective near-infrared spectroscopy provides a powerful tool for *in vivo* analysis of the mechanistic roles of these substances in living systems.

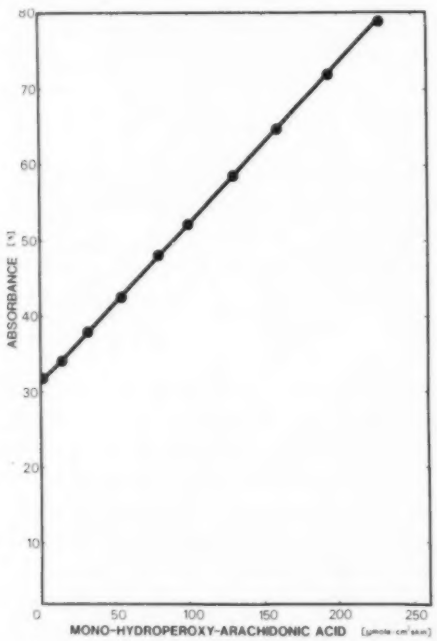

FIG. 3. Calibration curve drawn as percent absorbance at 1457nm versus varying concentrations of monohydroperoxy arachidonic acid (HPA). HPA was chosen as a standard from Holman and Edmonson, 1956, and because arachidonic acid shows the greatest decrease in concentration during aging in rat as compared with all other fatty acids *in vivo* (Hegner, 1980). Varying concentrations of HPA were dissolved in 1 ml toluene, and the recorded absorbance values were subtracted from a 1 ml toluene blank.

SUMMARY

A new method is described for the measurement of hydroperoxides and oxidized collagen in epidermal skin of mice, rabbits and man using reflective, near-infrared spectroscopy. Slight decreases in levels of hydroperoxides and oxidized collagen occurred during the non-senescent phase of life. On the other hand, dramatic increases occurred during the senescent phase. These increases were smallest in man, who has the most efficient defence system against peroxides and other active oxygens. Preliminary work suggests that significantly reduced levels of hydroperoxides and oxidized collagen can be obtained with antioxidant diet-supplementation in middle-aged mice (~90%, ~34%) and man (~15%, ~19%).

Acknowledgement—The Swedish Medical Research Council and Swedish Natural Science Research Council are acknowledged for their generous financial support. Evald Koitsalu and Drs. Gunnar Wettermark, Frederick Lund, Kurt Bergström, Mathias Uhlén and Ambjörn Ågren deserve special thanks for their help and advice.

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AGE-RELATED CHANGES OF ACTIVITY OF ACID PHOSPHATASE IN PHA-STIMULATED LYMPHOCYTES

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Abstract—Peripheral blood lymphocytes of 70-year-old individuals as well as spleen cells of 18-month-old Balb/c mice were characterized by diminished activity of acid phosphatase in relation to the activity of that enzyme in cells from young subjects. Simultaneously performed histochemical tests revealed that aging process in both species examined was accompanied by a reduction of the number of cells, disclosing the activity of acid phosphatase. Age-related differences with regard to the level of acid phosphatase became more pronounced after stimulation of cells with PHA. The decrease of acid phosphatase activity during aging is discussed in relation to the function of lymphocytes.

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INTRODUCTION

RECENTLY WE have found (Myśliwska *et al.*, 1983a) that activity of acid phosphatase (AP) in lymphocytes tends to decrease in senescence.

On the other hand, the level of activity of AP in lymphocytes undergoes variations following mitogenic stimulation (Myśliwska *et al.*, 1983b), which has been documented on lymphocytes of young subjects.

On the basis of these two facts there has arisen a question to what extent the aging process could interfere with the ability of lymphocytes to elevate the activity of AP upon mitogenic stimulation.

Additionally, in order to answer the question whether age-dependent decline of AP activity in lymphocytes is a more general phenomenon, we decided to examine the activity of that enzyme in splenocytes of mice representing different age groups.

By simultaneous application of biochemical and histochemical methods we also tried to examine whether the decline of AP activity in senescence reflects the diminution of the number of AP-positive cells—expressing activity of the enzyme, or if it is connected with a reduction of enzymatic potential of cells without alteration of their number.

(*Received* 26 *March* 1984)

MATERIALS AND METHODS

Blood donors

Fifteen young (aged between 20 and 35 years—mean age = 26 ± 8.8) and 15 old (above 70 years—mean age = 74 ± 6) volunteers were qualified to the experiments. Group of young subjects comprised 58% of males and 42% females. In the old group males constituted 32%, females 68%. Aged subjects inhabited an old peoples' home and remained under medical observation over a period of at least 6 months. During the last two months they did not receive medications, except vitamins.

Both groups were examined for chronic and acute diseases; tumors and autoimmune diseases have been excluded.

Mice

Sixty mice of Balb/c strain, males, have been divided into three groups—young (6–8 week) and 15- and 18-month-old—qualified as old. The mean survival for Balb/c mice, maintained in our laboratory was 20 months. Degree of senescence of mice was determined by adoption of grading score system introduced by Takeda *et al.* (1981). According to this classification, the group of 15-month-old mice corresponded to the second, and 18-month-old to the third degree of the advancement of age-related changes.

Isolation of human lymphocytes

Mononuclear cells were obtained by centrifugation on Lymphoprep (Nyegaard, Norway) gradient, Mononuclear cells were suspended in Minimal Essential Medium (MEM) (Biomed, Poland) supplemented with 20% calf serum (CS) (Biomed, Poland), L-glutamine (200 mM) (Sigma), penicillin (100 U/ml) and streptomycin (100 μg/ml).

Monocytes were removed by incubation of mononuclear cells in plastic tissue culture dishes for 1 h at 37°C. This procedure was repeated three times. Monocyte contamination was lower than 1%.

Isolation of mouse spleen cells

Spleen was cut into small pieces. The cells were obtained by teasing the organ fragments through the mesh. Erythrocytes were lysed with cold solution of NH_4CL (pH-7.2). For depletion of adherent cells the same procedure—incubation on plastic tissue culture dishes—as that used for human mononuclear cells, was applied.

PHA-stimulated cultures of human peripheral blood lymphocytes and mouse spleen cells

Lymphocyte and mouse spleen cell cultures were set in MEM medium to which calf serum was not added. Human cells were cultured at a concentration of 0.5×10^6/ml, mouse spleen cells at 1×10^6/ml. PHA (Wellcome) was added at concentration of 10 μl/ml to both kinds of cells. The concentrations of cells and PHA, according to the preliminary experiments, yielded the highest results in AP activity. Cells were incubated for 24, 48 and 72 h at 37°C in closed plastic Eppendorf tubes. After incubation cells were washed three times with phosphate buffered saline (PBS) and adjusted to the desired concentration.

Determination of AP activity

After incubating of cellular button with Triton—X 100 at 37°C for 30 min—cells were disrupted with a glas homogenizer. Cellular lysates were then centrifuged at 1000 × G for 15 min. Supernatant was removed and immediately assayed. Incubation medium was prepared according to Gillissen *et al.* (1973). P-nitrophenyl phosphate (Sigma) was used as a substrate. Incubation was carried out for 60 min at 37°C. Activity of AP was determined spectrophotometrically at $\lambda = 405$ nm. NaL and sodium-potassium tartrate inhibited activity of AP in a dose dependent manner.

Histochemical assay of AP

Histochemical reaction was performed according to Bancroft (1967). Slides were fixed in formol-calcium fluid and incubated in acetate buffer (pH-5.6) in presence of alpha naphtyl sodium phosphate (Sigma) as substrate. Fast Blue B was used as a coupler. The reaction product was visible in form of small granules scattered in cytoplasm.

Protein content

Protein content was determined according to the method of Lowry *et al.* (1951).

RESULTS

Activity of AP in resting and PHA-stimulated mouse spleen cells

The level of activity of AP in splenocytes of 15-month-old mice was indistinguishable from that of 8-week-old ones. Spleen cells of 18-month-old mice, however, exhibited lower activity of enzyme in comparison with cells of young mice (Figure 1).

After stimulation with T cell mitogen – PHA (Stobo, 1972), spleen cells of young mice reacted with an elevation of AP activity (Figure 1). In contrast, spleen cells of both groups of mice categorized as old did not produce alteration of AP activity following PHA stimulation.

The mitogen-induced elevations of AP activity in lymphocytes were calculated in relation to the values characteristic for non-stimulated cells in the course of the culture. It is noteworthy that activity of AP in non-stimulated spleen cells of all age groups showed similar tendency. There was a progressive reduction of the activity of enzyme which finally – at 72 hours of incubation – had dropped by as much as about 50% in relation to the initial values, measured immediately after isolation of cells (data not shown).

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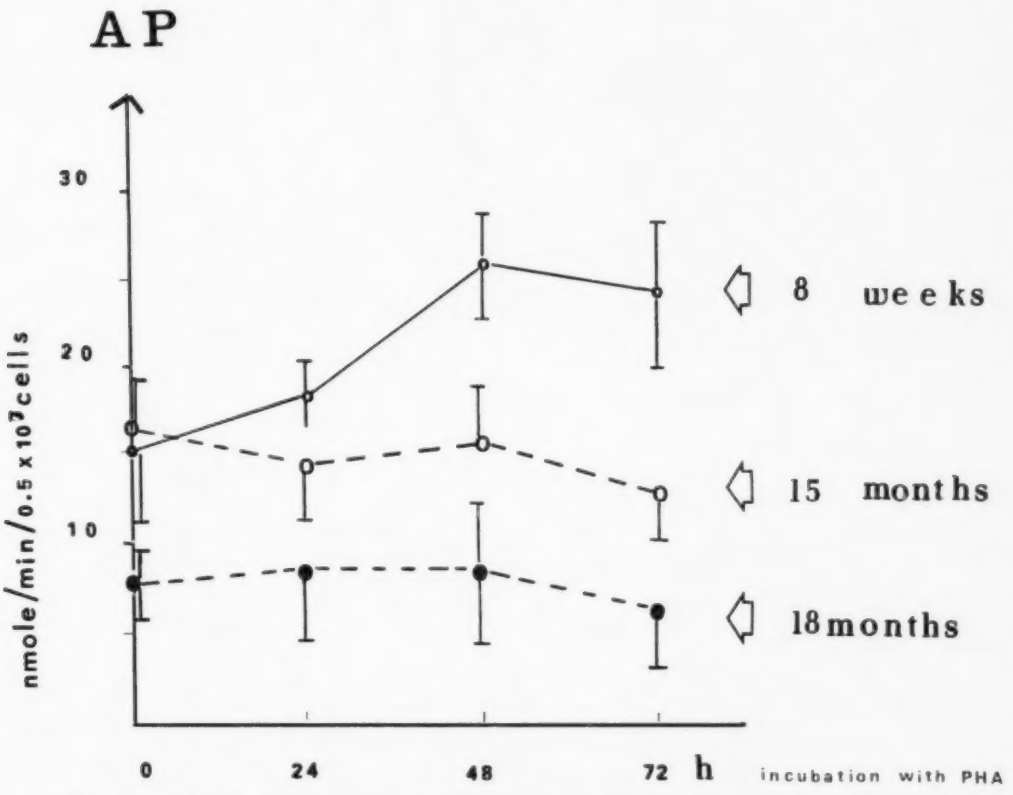

FIG. 1. Activity of AP in resting and PHA-stimulated mouse spleen cells. Each result represents arithmetic mean ± S.D. of measurements done on spleen cells from 15 mice.
Increase of AP activity in cells of young mice after 48 h of incubation with PHA is statistically significant ($p < 0.01$).

Thus, under resting conditions spleen cells of 15-month-old mice were equal with those of 8-week-old ones, in respect of AP activity. The same cells when stimulated with mitogen were not able to maintain the same level of AP activity as that characteristic for cells of young mice.

Comparison of the number of cells exhibiting activity of AP in histochemical method (Table 1) with the results of biochemical evaluations revealed that age-related phenomena in mouse spleen cells involve reduction of the number of cells characterized by the presence of AP. These results, however, do not allow one to establish whether the drop of AP activity observed in biochemical examination is exclusively due to the reduction of the number of cells disclosing the activity of AP or to the diminution of activity of AP by the cells equipped with this enzyme.

To see whether the differences in the capacity of spleen cells of young and 15-month-old mice to raise the AP activity following PHA stimulation could be detected in the early phase of mitogenic stimulation, short term incubation of spleen cells with PHA was applied.

TABLE 1. PERCENTAGE OF CELLS WITH HISTOCHEMICALLY DETECTABLE ACTIVITY OF AP IN POPULATION OF RESTING AND PHA-STIMULATED MOUSE SPLEEN CELLS.

Age	*Non-stimulated cells*	*PHA-stimulated cells*		
		24 h	*48 h*	*72 h*
6–8 weeks	58 ± 11	78 ± 11	80 ± 6.7	60 ± 5.5
15 months	35 ± 8	50 ± 6.6	35 ± 8.8	30 ± 15

Each result represents arithmetic mean ± S.D. of evaluations done on cells of 15 mice.

Increase of the percentage of cells with histochemically detectable activity of AP is statistically significant ($p < 0.1$) only for 48 h PHA-stimulated cultures of spleen cells from young mice.

TABLE 2. ACTIVITY OF AP IN RESTING AND PHA-STIMULATED HUMAN BLOOD LYMPHOCYTES.

	Non-stimulated cells		*PHA-stimulated cells—24 h*		*PHA-stimulated cells—48 h*		*PHA-stimulated cells—72 h*	
age	*nmole/min/ 5×10^5 cells*	*nmole/min/mg protein*	*nmole/min/ 5×10^5 cells*	*nmole/min/mg protein*	*nmole/min/ 5×10^5 cells*	*nmole/min/mg protein*	*nmole/min/ 5×10^5 cells*	*nmole/min/mg protein*
20–35	4.8 ± 1.9	62.6 ± 24.7	3.9 ± 2.4	59.1 ± 20.1	8.4 ± 3.8	108.8 ± 49	7.2 ± 3.0	92.8 ± 38.4
70	2.4 ± 1.7	25.1 ± 13.2	1.7 ± 0.9	18.1 ± 11.1	3.0 ± 1.8	30.9 ± 18.8	2.7 ± 1.4	27.0 ± 15.5

Each result represents arithmetic mean ± S.D. of measurements done on lymphocytes of 15 individuals.

Increase of AP activity is statistically significant only in 72 h PHA-stimulated cultures of young donors ($p < 0.02$).

As shown in Figure 2, short term incubation with PHA had effect only on spleen cells of young mice. It resulted in marked increase of AP activity emerging at 40 min of incubation, followed by a return to the values characteristic for non-stimulated cells. This elevation did not occur in spleen cells of 15-month-old mice.

Thus, short term stimulation test with PHA was sufficient in detecting age-related differences in metabolic potential of spleen cells.

Activity of AP in resting and PHA-stimulated human blood lymphocytes

Peripheral blood lymphocytes of over-70-years-old subjects displayed weaker activity of AP than those of persons aged between 20 and 35 years. PHA stimulation resulted in elevation of AP level, occuring at 48 and 72 h of culture, which was, however, statistically significant only in cultures of cells of young persons (Table 2).

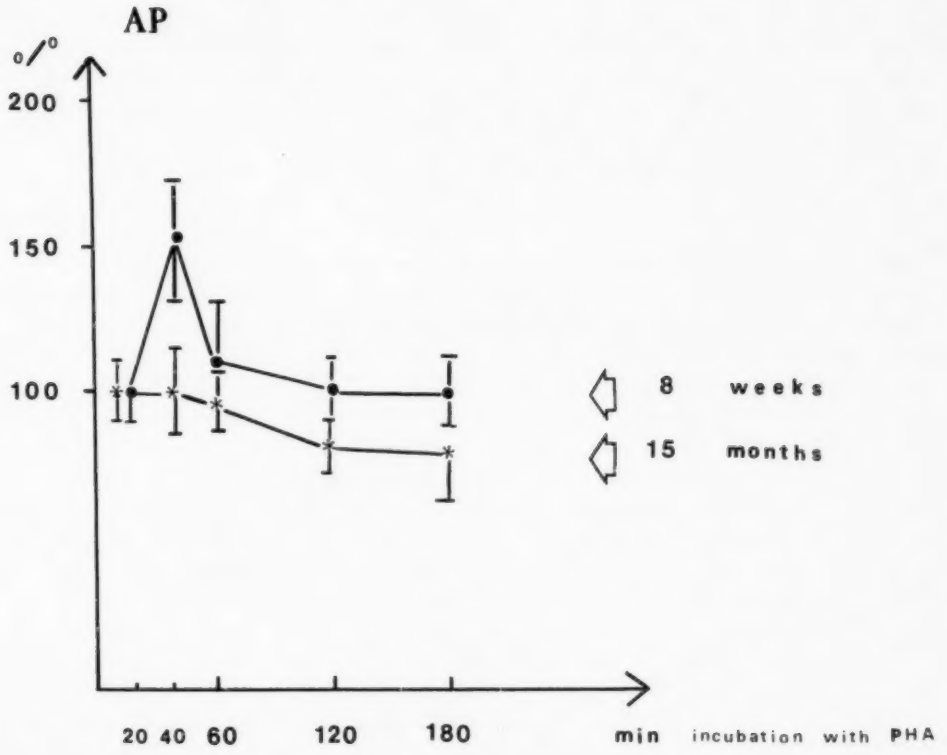

FIG. 2. Changes in activity of AP in mouse spleen cells following their short term incubation with PHA.
Results are expressed as a percentage of values of AP activity found in cells maintained under the same conditions, but without PHA, assumed as 100%.
Each point represents arithmetic mean ± S.D. of results obtained on cells from 7 mice.
The increase of AP activity in spleen cells of young mice after 40 min of incubation with PHA is statistically significant ($p < 0.05$).

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TABLE 3. PERCENTAGE OF CELLS WITH HISTOCHEMICALLY DETECTABLE ACTIVITY OF AP IN POPULATION OF RESTING AND PHA-STIMULATED HUMAN BLOOD LYMPHOCYTES.

age (years)	*non-stimulated cells*	*PHA-stimulated cells*		
		24 h	*48 h*	*72 h*
20–35	45 ± 5.5	55 ± 11	68 ± 20	77 ± 8.5
70	28 ± 7.2	36 ± 10	47 ± 15	55 ± 6.2

Each result represents arithmetic mean ± S.D. of evaluations done on lymphocytes of 15 persons.

The increase of percentage of cells displaying AP activity is statistically significant ($p < 0.001$) in case of 72 h PHA-stimulated cultures of cells from young and aged donors.

Since the protein content in cells of old individuals was higher than that of young persons, the decrease of AP activity in cells from old persons became more pronounced when the results were expressed in relation to the protein content.

Age-related decline in activity of AP in human lymphocytes was, as in mouse spleen cells, associated with a drop of the number of cells expressing activity of AP (Table 3).

Thus, spleen cells of mice and human blood lymphocytes showed tendency to reduce with advancing age the activity of their AP and to diminish the number of cells capable of expressing the positive histochemical reaction.

DISCUSSION

Results of our investigations have confirmed that the aging process is accompanied by a decline in activity of one of the hydrolytic enzymes—acid phosphatase. This tendency showed both human peripheral blood lymphocytes as well as mouse spleen cells depleted of adherent cells.

Initial manifestation of the age-related alterations of the AP activity in lymphocytes was the inability of these cells to raise the level of AP activity following mitogenic stimulation. Under resting conditions, however, these cells still preserved their capacity to maintain the level of AP in the range of values indistinguishable from those characteristic for cells of young subjects. Further progression of impairment of lymphocytes with regard to the possibility of maintaining a sufficiently high level of AP was manifested by a reduction of the AP activity not only after stimulation but also in a resting state.

Decline of AP activity in human blood lymphocytes and mouse spleen cells was detectable by both biochemical and histochemical assays. This fact implies that age-related decline in activity of AP in lymphocytes could result partially from the limitation of the number of cells expressing AP activity—from the quantitative changes in population of lymphocytes—and partially from the qualitative changes. These latter changes could be connected with a weakening of enzyme activity in cells actually displaying histochemically detectable activity of AP.

The application of different substrates in bio- and histochemical assay does not allow to establish unequivocally which of the mechanisms mentioned is more responsible for the changes connected with age.

Detection with histochemical method of activity of AP in human blood lymphocytes

serves as diagnostic tool in some proliferative immunological disorders, as this enzyme is regarded as a marker of T cell lineage origin (Pangalis *et al.*, 1978; Stein *et al.*, 1976). Our results indicate that every such diagnosis should be treated cautiously and take into account age-related differences in the number of cells reacting positively in histochemical assay.

Age-related fluctuations of activity of AP in different cells and tissues have been the subject of several studies. In spite of this we have not come across any investigations dealing with the activity of that enzyme in lymphocytes in the course of aging process, with exception of our previous studies (Myśliwska *et al.* 1983a).

The examinations done on AP activity during aging brought contradictory results. All possible variations—increase (Hayflick, 1980; Sleyster *et al.*, 1980), decrease (Bertolini, 1962; Kurnick *et al.*, 1962), and lack of changes in the level of AP activity (Palatroni *et al.*, 1980; Wilson *et al.*, 1982) during aging—have been described.

This discrepancy could have been due to the use of various parameters for the expression of measurements in biochemical assay. As a basis for the expression of AP activity: wet weight, protein, nitrogen and DNA content, dry weight, and the cell number have been used (Wilson, 1973). In our examinations age-related decline of AP activity in lymphocytes was more intensive when calculated in relation to the protein content.

Another reason for the discrepancy in the results of various authors could lie in testing and comparing animal and human cells of non-corresponding ages.

Many times the details concerning the survival and mortality rate of the examined animals have not been given. For example, both 24 to 30-month as well as 15-month-old mice have been considered as old. Among the studied rats, 18 to 27-month-old have been determined as old, but also 6 to 7-month-old have been chosen as examples of old age (Wilson, 1973).

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Age 18 months for mice have been found in our preliminary experiments as decisive for the fall of activity of AP in spleen cells of the great majority of mice. Similarly, age of 70-years for humans was chosen on grounds of our preliminary experiments. These experiments revealed that at about this age the lymphocytes of almost 90% of persons were characterized by a decline of activity of AP.

Our previous results (Myśliwska *et al.*, 1983b) revealed the correlation between the magnitude of AP activity in lymphocytes and the height of their spontaneous proliferative activity. Barr *et al.* (1976) showed that the presence of AP activity is predominantly a feature of blast cells. These data together suggest that AP can play a role in the process of cellular division. It also implies that altered activity of AP could at least partially contribute to the age-related impairment of mitogenic reactivity of lymphocytes.

We have also found a correlation between the magnitude of cytotoxic reactivity of lymphocytes in ADCC and NK reactions and AP activity in human peripheral blood lymphocytes during aging (Myśliwska *et al.*, 1983a). It is possible that dysfunction of lymphocytes in cytotoxic reactions during aging could also be partially explained by the decline of AP activity.

Since activity of AP resides predominantly in T cells (Poore *et al.*, 1981; Myśliwska *et al.*, 1983b), being evenly distributed among functionally different T cell subsets (Bernard *et al.*, 1983; De Wacle *et al.*, 1983), it would also be conceivable that the age-related impairment of broad spectrum of T cell functions is associated with the decline of AP activity.

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IN VIVO DNA SYNTHESIS IN THE DIETARY RESTRICTED LONG-LIVED RAT

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CONTROLLED DIETARY restriction in rodents has provided one of the most useful animal models in which to study the mechanisms of aging. Although many workers using a variety of nutritional manipulations have reported a significant extension in maximum lifespan, the molecular mechanisms delaying age related physiological deterioration and associated pathological lesions are unknown (Ross, 1961; Merry and Holehan, 1979; Masoro, 1980). An extension of the lifespan has been observed in rodents in spite of widely differing experimental conditions, species and strain of animal, composition, and caloric value of the diet (Ross, 1978; Frolkis, 1982). There appears to be a direct link between the extent to which nutrition is limited, the initiation of the restricted diet and an increase in the lifespan (Ross, 1959; Weindruch and Walford, 1982). The major effect of dietary restriction is on reducing the rate of mortality at the later stages of life and not by improving the maintenance and survival of young animals. It is noteworthy that food restriction is as effective in prolonging the life of long-lived SPF maintained rats as it is in rats maintained in conventional facilities (Yu *et al.*, 1982).

During early development and growth, rates of DNA and RNA synthesis supersede breakdown until the limit of growth is reached and a steady state is established. During aging the steady state is destroyed (Petricevic *et al.*, 1983). The finding of a reduced DNA content in liver or muscle of animals receiving restricted nutrition has been well documented (Williams, 1961; Fukuda and Sibatani, 1953; Graystone and Cheek, 1969). Barrows and Kokkonen (1978) have reported an increase in the DNA per unit wet weight in liver from fasting rats or from animals fed *ad libitum* a 4% protein diet, indicating a reduction in cell size with underfeeding (Ross, 1969). Many of the observations on the effect of nutrition on DNA synthesis have concentrated on young or old animals. In recognition of this a lifetime study of *in vivo* DNA synthesis was undertaken in rats maintained on a restricted diet.

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(*Received* 18 *April* 1983, *Revised* 12 *September* 1984)

METHODS AND PROCEDURE

Male Sprague-Dawley (CFY strain) rats were weaned at 21 days and randomly allocated to control or experimental groups. All animals were caged individually and allowed unrestricted access to water. Growth was retarded in the dietary restricted animals by limiting intake of the pelleted diet, (Rat Breeder diet, E.H. Bradshaw & Sons Ltd., Driffield, England) such that the body weight was maintained at 50% that of the age-matched *ad libitum* fed rats. The animal house temperature was maintained at 21 ± 1°C, humidity within the range 50–70%, and a light regimen of 12 hrs light/24 hrs. The restricted diet was preweighed and supplied at 11.00 hrs colony time each day. Cumulative mortality was recorded and expressed in 50 day cohorts for *ad libitum* fed and dietary restricted rats. The restricted feeding protocol employed in the present work has been shown in previous studies to increase maximum and mean lifespan by greater than 40% without inhibiting growth, sexual maturation or resulting in infertility (Merry and Holehan, 1981).

An index of *in vivo* DNA synthesis was determined in liver, kidney, heart, abdominal skin, small intestine and thymus in five fully-fed and dietary restricted rats at regular intervals from weaning to 1115 days. DNA synthesis was assessed by the incorporation of ^{3}H-thymidine into DNA according to the method of Henderson, Fischel, and Loeb (1971). Animals were injected I.P. with a dose of 1μCi/10 gm body mass of ^{3}H-thymidine (sp. act. 45 Ci/mmol) dispersed in 0.5ml of isotonic saline at 09.00 hrs colony time prior to killing 2 hours later. Tissue content of DNA was determined according to the method of Burton (1956); tissue RNA was assayed by its absorption at 260nm by the method of Munro and Fleck (1966); protein was determined by the biuret reaction.

Total DNA, protein and RNA were not determined for abdominal skin and intestine, for biochemical procedures were performed on representative tissue samples.

Statistics

Regression lines derived from the Gompertz plots were analyzed for homogeneity of residual variances, significant differences in slope and intercept according to the method of Snedecor and Cochran (1967). The four ratios dpm/DNA, RNA/DNA, protein/DNA, and protein/RNA were analysed by a two-way factorial analysis of variance according to the algorithms in the Genstat 4-01 computer programs published by the Lawes Agricultural Trust (Rothamsted Experimental Station, 1977). Specific comparisons of group means were made according to the contrast method of Scheffé (Harris, 1975).

RESULTS

Mortality

Comparison of the age-specific mortality rate against age for fully-fed and dietary restricted male rats held at 50% control growth rates revealed a significant ($p < 0.001$) difference in the slope of the regression line. An extension of 42% in the maximum lifespan was seen in the experimental rats. A higher initial rate of mortality was revealed in the restricted animals if the regression lines of the Gompertz plots were extrapolated to day 0, (Figure 1).

DNA, RNA, and protein contents

The slower growth rate of the dietary restricted rats when compared with *ad libitum* fed animals was reflected in the lower DNA, RNA, and protein contents of liver, kidney and heart with age (Table 1, Figure 2), and these organs showed a similar profile in their response to dietary restriction. A decrease in the RNA preceded any effect on protein or DNA in these organs. In liver and heart little effect was observed in DNA over the first 7 days of restricted feeding while in kidney this period was extended to 14 days. After approximately 40 days of restricted feeding (61 days of age), DNA, RNA and protein levels stabilized in heart and kidney at about 40–60% of control values in subsequent age-matched comparisons. The effect of dietary restriction was more pronounced in liver, with DNA, RNA, and protein stabilizing between 20–40% of control values.

The growth of the thymus gland as shown by DNA, RNA, and protein was severely retarded in the experimental animals; with relative DNA, RNA, and protein content being between 10–25% of control levels at 35–60 days. After 100 days thymus tissue from *ad*

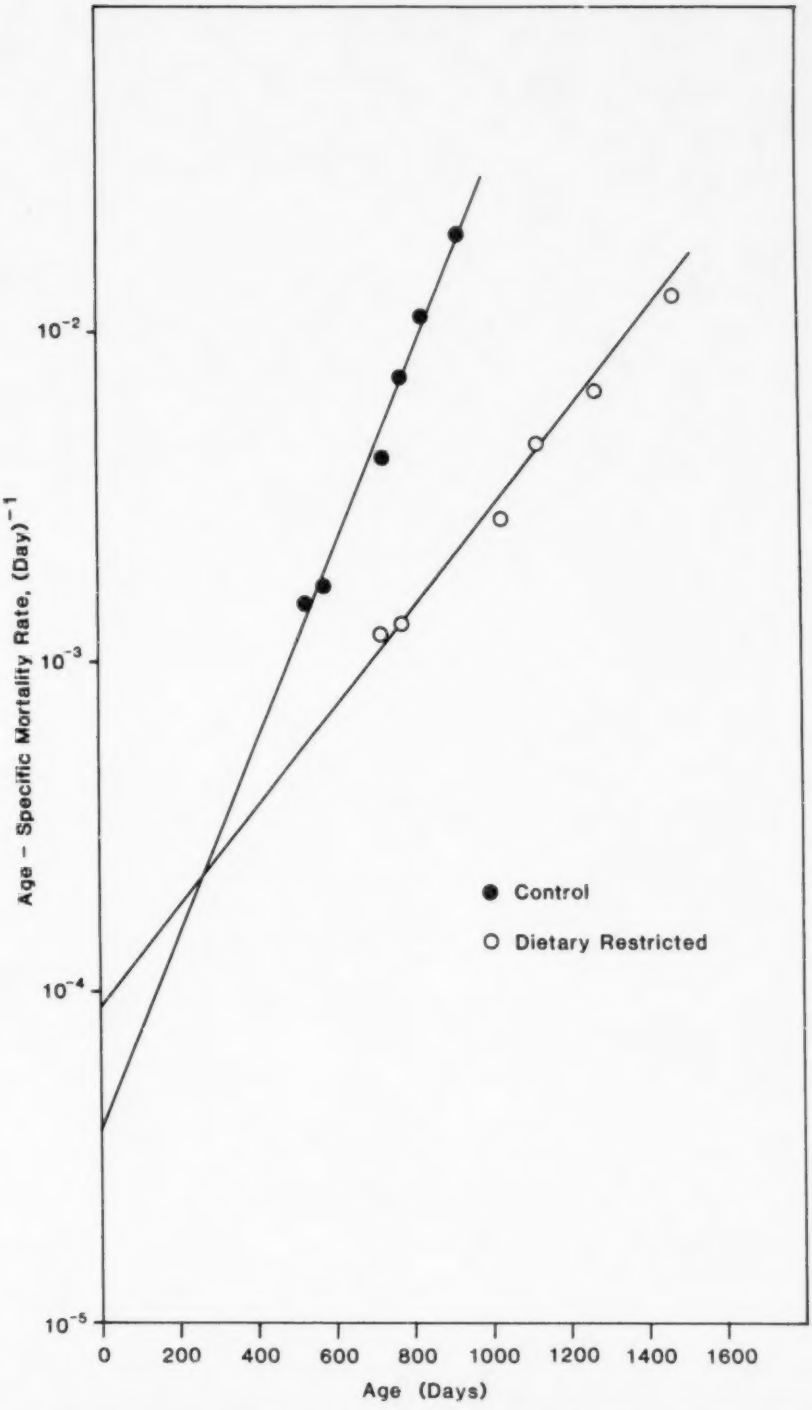

FIG. 1. Log_{10} age-specific mortality rates for *ad libitum* fed (●) and dietary restricted male rats (○).

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TABLE 1. DNA, RNA, AND PROTEIN CONTENT OF LIVER, KIDNEY, HEART AND THYMUS FROM MALE RATS FED *AD LIBITUM* OR A RESTRICTED DIET THROUGHOUT POSTWEANING LIFE.

Age (days)	TOTAL DNA mg		TOTAL RNA mg		TOTAL PROTEIN mg	
	AL	DR	AL	DR	AL	DR
Liver[1]						
21	3.23 ± 0.26	–	4.46 ± 0.35	–	382.3 ± 52.0	–
28	4.55 ± 0.30	4.34 ± 0.57	30.10 ± 1.26	7.79 ± 0.87***	1323.1 ± 23.7	542.9 ± 83.8***
35	9.43 ± 0.69	3.72 ± 0.10**	27.09 ± 1.13	6.75 ± 0.26***	2011.3 ± 204.4	706.8 ± 34.3**
60	20.40 ± 1.82	5.92 ± 0.42**	47.70 ± 1.50	12.35 ± 0.74***	3818.6 ± 244.6	1006.2 ± 36.9***
180	15.24 ± 0.65	5.84 ± 0.27***	30.25 ± 1.87	10.72 ± 0.72***	9590.3 ± 700.4	2580.5 ± 82.1***
265	13.70 ± 1.83	4.36 ± 0.47**	41.35 ± 1.75	12.00 ± 3.36***	9020.0 ± 580.5	2174.6 ± 80.1***
360	15.18 ± 1.51	5.59 ± 0.78**	40.02 ± 3.77	11.59 ± 0.41**	9318.9 ± 692.7	2744.2 ± 117.2***
457	23.40 ± 1.44	9.76 ± 0.47***	33.30 ± 1.94	9.34 ± 0.42***	8561.6 ± 752.2	3008.0 ± 212.7***
756	11.46 ± 0.66	4.16 ± 0.40***	24.83 ± 3.16	12.62 ± 0.83**	6390.3 ± 759.3	2643.4 ± 659.2***
Kidney[1]						
21	4.42 ± 0.22	–	2.34 ± 0.03	–	131.3 ± 11.4	–
28	3.92 ± 0.23	3.85 ± 0.33	2.87 ± 0.08	1.92 ± 0.10**	204.0 ± 6.7	145.9 ± 12.2**
35	1.96 ± 0.19	1.97 ± 0.03	4.01 ± 0.09	1.74 ± 0.06***	250.1 ± 8.7	134.4 ± 7.4***
60	7.02 ± 0.47	4.19 ± 0.09**	3.71 ± 0.16	1.40 ± 0.10***	466.2 ± 41.0	166.2 ± 14.6***
180	4.25 ± 0.29	2.39 ± 0.11***	6.39 ± 0.40	3.16 ± 0.11***	596.7 ± 38.6	276.5 ± 25.5***
265	7.11 ± 0.24	2.84 ± 0.27***	7.48 ± 0.90	2.67 ± 0.36**	703.4 ± 55.0	291.6 ± 13.0**
360	4.54 ± 0.57	2.74 ± 0.23*	7.19 ± 0.78	3.18 ± 0.31**	712.5 ± 120.5	418.1 ± 37.9*
457	7.94 ± 0.75	3.86 ± 0.53	5.95 ± 1.44	2.94 ± 0.63	740.4 ± 50.4	384.5 ± 23.6***
756	8.34 ± 2.04	3.07 ± 0.20*	6.52 ± 0.95	3.14 ± 0.22*	876.2 ± 101.3	428.4 ± 29.4**

Heart[1]						
21	0.40 ± 0.05	–	0.48 ± 0.03	–	65.9 ± 7.8	–
28	0.46 ± 0.04	0.42 ± 0.09	0.80 ± 0.02	0.25 ± 0.01**	91.3 ± 7.3	51.0 ± 5.7**
35	1.00 ± 0.12	0.68 ± 0.03*	1.24 ± 0.06	0.40 ± 0.10**	81.4 ± 10.6	25.4 ± 3.1***
60	0.86 ± 0.05	0.43 ± 0.05***	0.97 ± 0.05	0.39 ± 0.02***	207.8 ± 15.5	84.6 ± 4.7***
180	0.74 ± 0.13	0.57 ± 0.05	0.71 ± 0.05	0.39 ± 0.02***	244.4 ± 32.7	90.6 ± 2.9**
265	1.08 ± 0.09	0.44 ± 0.03***	1.44 ± 0.13	0.57 ± 0.05***	355.6 ± 48.3	131.1 ± 10.0*
360	1.02 ± 0.11	0.51 ± 0.08**	0.78 ± 0.12	0.36 ± 0.03*	404.3 ± 62.0	134.9 ± 9.6*
457	1.03 ± 0.08	0.58 ± 0.05**	1.41 ± 0.06	0.79 ± 0.04***	331.5 ± 14.4	189.3 ± 9.3***
756	1.25 ± 0.10	0.88 ± 0.07*	0.86 ± 0.11	0.54 ± 0.05*	391.4 ± 29.6	234.7 ± 11.9**
Thymus[2]						
21	0.31	–	1.33	–	5.5	–
28	0.72	0.21	2.25	0.90	6.4	3.4
35	1.13	0.12	3.57	0.51	9.8	2.5
60	1.29	0.20	4.19	0.80	17.7	4.9
180	0.43	0.17	1.65	0.64	6.8	3.8
265	0.53	0.16	1.17	0.44	6.4	5.1
360	0.04	0.10	0.14	0.23	6.8	5.2
457	0.17	0.21	0.19	0.26	9.6	7.2
756	0.03	0.03	0.09	0.08	2.6	2.5

Data analysed by two-way factorial ANOVA. Specific comparisons of group means were made by the method of Scheffé *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

1. 5 animals per age per group: AL = ad libitum: DR = dietary restricted.
2. 5 animals per age group but tissue pooled.

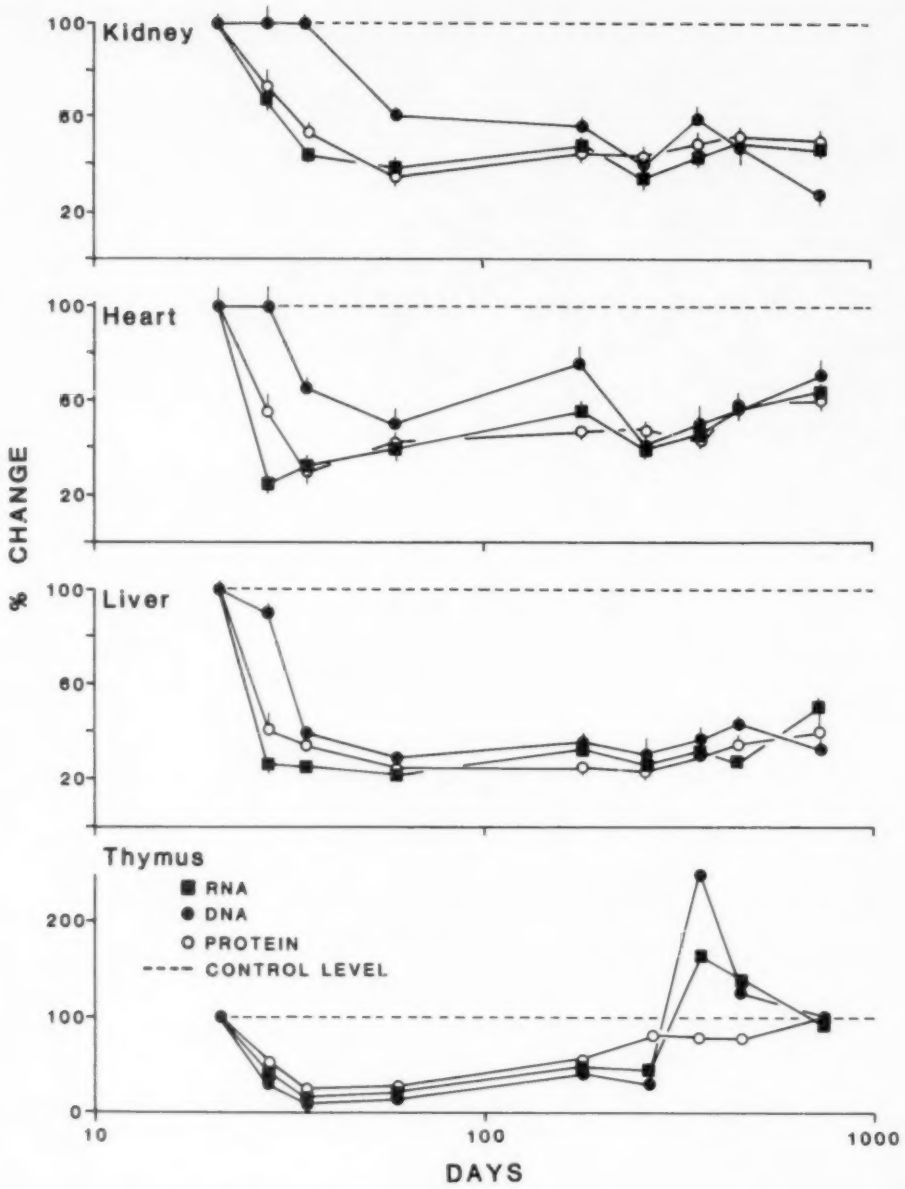

FIG. 2. Relative change in DNA, RNA and protein tissue content for kidney, heart, liver, and thymus for male rats maintained on a restricted diet.

libitum fed rats showed a rapid involution which was not seen in thymus tissue from animals on a restricted diet. Consequently RNA and protein content exceeded control values at ages beyond one year. The DNA content of thymus tissue from restricted rats gradually increased to control levels by 2 years of age.

DNA synthesis

Liver and kidney tissue from rats on the restricted diet showed a significant ($p < 0.001$) reduction in the incorporation of ^{3}H-thymidine into DNA. A significant diet, age interaction ($p < 0.001$) was observed for the rate of DNA synthesis in these two tissues, restricted feeding eliminated the prepubertal peaks of DNA synthesis observed between 21–60 days in the *ad libitum* fed rats. After 100 days no significant effect of underfeeding on the rate of DNA synthesis was observed in these tissues (Figure 3).

The rate of DNA synthesis in heart and abdominal skin was significantly decreased ($p < 0.001$) in animals on restricted feeding. There was a significant age effect ($p < 0.001$) observed for DNA synthesis in heart and abdominal skin but no significant diet, age interaction. The developmental peak of DNA synthesis was reduced but not eliminated in these two tissues. Because of the large variances associated with the means, no significant differences could be demonstrated between age matched means for *ad libitum* fed and dietary restricted animals by the conservative Scheffé contrast method.

A high rate of DNA synthesis was observed in the small intestine and a significant effect of age ($p < 0.001$) was evident in this tissue. No inhibition of ^{3}H-thymidine incorporation into DNA was observed in the small intestine from animals maintained on a restricted diet.

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Thymus tissue was pooled within age and diet groups and consequently no estimate of the variance of DNA synthesis within groups could be made. The mean levels of DNA synthesis were higher in thymus tissue from dietary restricted animals at all ages from 28 to 365 days with the exception of 60 days, which is the time of peak thymus size and DNA synthesis in fully-fed animals.

RNA/DNA Ratio

A significant age effect ($p < 0.001$) was observed in the RNA to DNA ratio in all tissues. In liver, kidney, abdominal skin and heart, dietary restriction had a significant effect in reducing the RNA to DNA ratio, while in the small intestine no effect of diet on this ratio could be demonstrated. Liver, heart, and kidney revealed a significant interaction ($p < 0.001$) between age and diet reflecting the greater reduction of RNA content in dietary restricted rats compared to DNA content during the first 180 days of life. Thymus tissue from dietary restricted rats showed a higher RNA to DNA ratio during the first 200 days of life than tissue from control animals (Figure 4).

Protein/RNA Ratio

All six tissues showed a significant ($p < 0.001$) age related increase in the protein to RNA ratio. No overall significant effect of diet could be demonstrated in the protein to RNA ratio for liver, heart, kidney, or intestine although the mean contrast at 365 days for kidney did reveal a significantly higher ratio ($p < 0.01$) in fully fed rats. A significant age, diet interaction ($p < 0.001$) was observed for the protein/RNA ratio in abdominal skin.

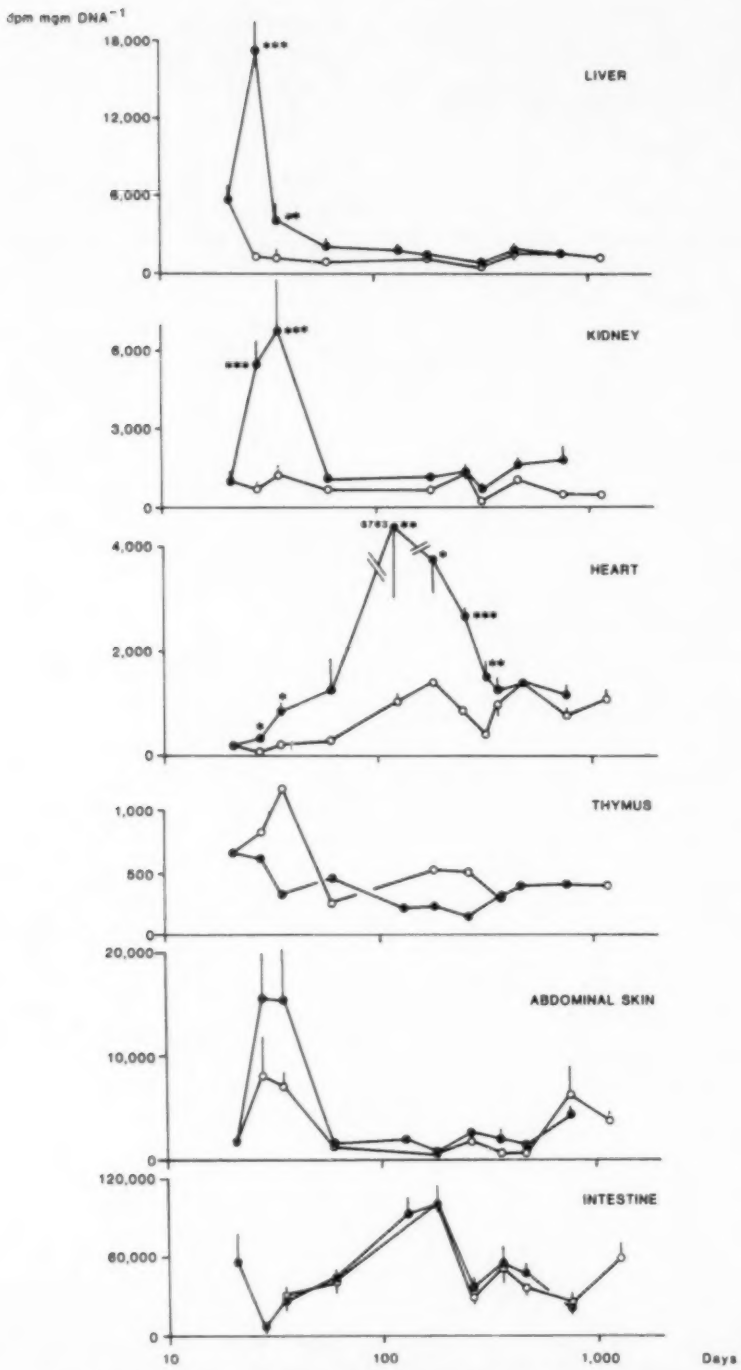

FIG. 3. Incorporation of ^{3}H-thymidine mgm DNA^{-1} in liver, kidney, heart, thymus, abdominal skin, and small intestine in male rats fed *ad libitum* (●) and maintained on a restricted diet (○). Each point is the mean and s.e.m. for 5 animals. The mean value shown for thymus tissue is derived from a tissue pool from 5 animals. Dietary restricted animals compared to the age-matched *ad libitum* fed rats; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

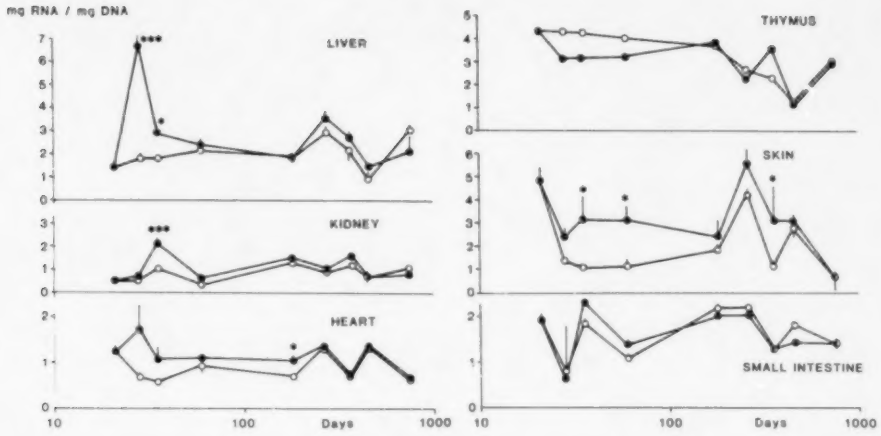

FIG. 4. RNA/DNA ratio with age for liver, kidney, heart, thymus, abdominal skin, and small intestine in male rats fed *ad libitum* (●) or a restricted diet (○). The mean and s.e.m. is shown for 5 animals except for thymus tissue where the mean value only is given for a tissue pool from 5 rats. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

This resulted from the very high ratios observed at 2 years of age in abdominal skin from both fully fed and dietary restricted animals. The protein to RNA ratio in thymus was marginally higher in thymus from dietary restricted rats up to 265 days, after which the ratio fell below the values observed in tissue from fully fed animals (Figure 5).

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Protein/DNA Ratio

A significant effect of age ($p < 0.001$) was seen in the protein to DNA ratio with age in all six tissues studied. As a general observation there was an increase in the protein to DNA ratio with age in all tissues and a significant reduction in the ratio ($p < 0.001$) for liver, heart, kidney, and abdominal skin in dietary restricted rats. A significant interaction of diet and age was demonstrated for liver ($p < 0.005$) and kidney ($p < 0.001$) in the protein to DNA ratio. Contrasts of paired means in the respective tissues revealed significant

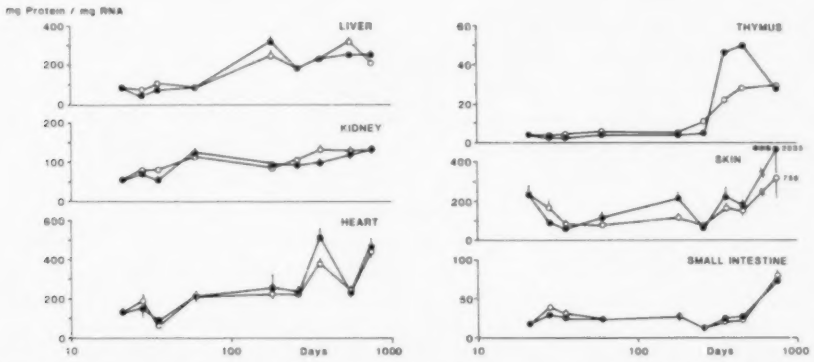

FIG. 5. Protein/RNA ratio with age for liver, kidney, heart, thymus, abdominal skin, and small intestine in male rats fed *ad libitum* or a restricted diet. All other details the same as for Fig. 4.

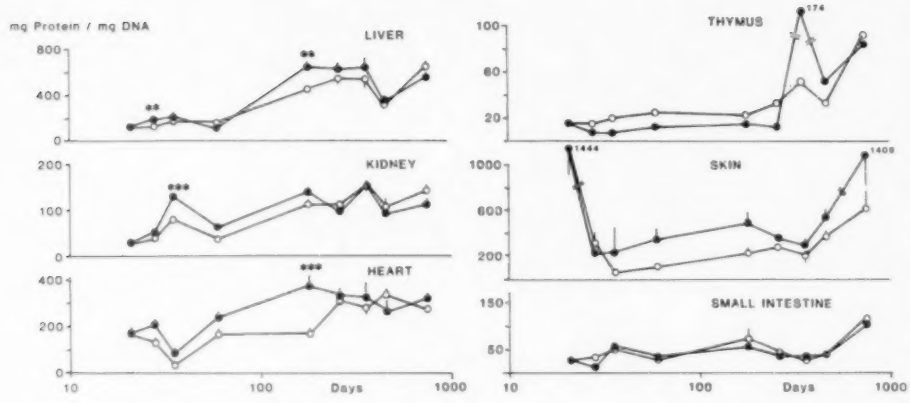

FIG. 6. Protein/DNA ratio with age for liver, kidney, heart, thymus, abdominal skin, and small intestine in male rats fed *ad libitum* or a restricted diet. All details the same as for Fig. 4.

differences in this ratio up to 180 days of age. In thymus tissues the protein to DNA mean ratios were higher in dietary restricted rats when compared to fully fed animals at all ages except 365 and 457 days of age (Figure 6).

DISCUSSION

Food restriction in a variety of forms has proved to be the most effective and reproducible procedure for decreasing the rate of aging (Sacher, 1977; Comfort, 1979). The findings of the present study support this general conclusion, and the extension in maximum lifespan observed in CFY Sprague Dawley rats (42% extension, maximum lifespan 1500 days) is comparable to that reported by Yu *et al.* (1982) for SPF maintained Fischer 344 rats (49% extension, maximum lifespan 1435 days) using a similar degree of dietary restriction.

A comparison of the Gompertz plot (Figure 1) with that published by Sacher (1977) for the pooled data from Berg and Simms (1960), Ross (1959), and Kimeldorf (1966) revealed a serious discrepancy for the day 0 mortality rates of both control and dietary restricted rats. The extrapolated mortality rate at birth for the control Gompertz line for the pooled data was $2.7 * 10^{-6}$ day^{-1} compared to $3.7 * 10^{-5}$ day^{-1} recorded in this study. This gave a doubling time of the rate of mortality (T_d) of 66 days in the analysis of Sacher (1977) which he commented was remarkably low in comparison to 100 days for the laboratory mouse, and longer doubling times for all other rodent species (Sacher and Staffeldt, 1972). The T_d calculated for the *ad libitum* fed rats in this study is 102 days. This anomaly was difficult to reconcile in view of the similar mortality profiles for control and experimental rats observed in the two studies. Recalculation of the T_d from the data of Ross (1959) did not substantiate the figure reported by Sacher (1977) and gave a value of 99 days for fully-fed animals. Restricted feeding decreased the T_d in CFY rats from 102 to 203 days while in the study of Ross (1959), restricted feeding of diet D (a diet similar in composition to the one used in this work), gave a T_d value of 210 days. Confirmation of the reproducibility of mortality rates between laboratories can be seen from the data of Yu *et al.* (1982) which

gives a value of 104 days for the T_d of *ad libitum* fed rats and a T_d of 189 days for rats dietary-restricted after 42 days of age.

In agreement with the findings of Graystone and Cheek (1969) for liver, muscle, and cerebrum, it was observed that DNA, RNA, and protein content of liver, kidney, heart and thymus were reduced in animals on restricted feeding. The finding of a reduced DNA content in liver or muscle of animals receiving restricted nutrition has been reported by Fukuda and Sibatani, (1953) and Williams (1961). The greatest reduction of DNA, RNA, and protein content was recorded for the liver and in all tissues a decrease in RNA content preceded any change in protein or DNA. Wannemacher *et al.* (1971) have reported a good correlation between the rate of hepatic protein synthesis *in vivo* and the cellular protein content of the liver. Rates of protein synthesis both *in vivo* and *in vitro* were found to be directly correlated with the hepatic concentrations of individual amino acids.

In kidney no significant change in DNA content was observed until after two weeks of restricted feeding, however, during the first 100 days of life the rate of DNA synthesis in both kidney and liver was very sensitive to underfeeding. Dallman and Manies (1973) reported that within 12 hours of initiation of a low protein diet (3.5% protein) fed *ad libitum* to 28-day-old-rats, DNA synthesis and the activity of thymidine kinase were decreased to less than half control values. In contrast, incorporation of ^{14}C-orotate into RNA increased to about double the control value and the uridine kinase activity became significantly increased by 2 days. Concurrent with the inhibition of DNA synthesis is a retardation of the ploidy changes observed with age in liver from fully-fed rats (Gahan *et al.*, 1984; Frolkis, 1982; Enesco and Samborsky, 1983). In control rats between 10–41 days there is a marked increase in the number of liver cells associated with a doubling of the DNA content per nucleus and an increase in cell size (Fukuda and Sibatani, 1953). The peak in the rate of DNA synthesis observed in liver from *ad libitum* fed rats between 21 and 35 days of age is in agreement with this observation, for 95% of ^{3}H-thymidine is incorporated into nuclear DNA and only 5% into mitochondrial DNA, the synthesis of which is increased by protein deficiency (Dallman and Manies, 1973). The present findings are in agreement with Winick and Noble, (1966) that postweaning dietary restriction implemented before 65 days of age reduced cell division in the liver by inhibiting DNA synthesis.

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Individual tissues respond in differing ways to the restricted diet and while the rate of DNA synthesis is decreased in liver, kidney, heart, thymus, and abdominal skin, no effect of undernutrition was observed on the rate of DNA synthesis in the rapidly dividing cells of the small intestine. This finding is in contrast to that of Munro (1970) who observed diminished uptake of ^{32}P into the DNA of the intestinal mucosa of protein-depleted rats. A protein free diet was found to retard the cycle of cell division and migration up the intestinal villus without affecting mean cell protein or RNA content (Platt, Heard and Stewart, 1964). Confirmation of the observation on mean cell size and RNA content stability in the intestinal mucosa under restricted feeding conditions was seen in the protein/DNA and RNA/DNA ratios recorded in the present study. The lack of response of the cells of the small intestine to the restricted feeding regime may reflect their anatomical position and the availability of amino acids and dipeptides resulting from the action of trypsin and peptide hydrolases in the brush border and cytosol of the mucosal cells. The effect of underfeeding on the thymus appears to restrict cell number with cell size and RNA content being increased during the first year of life.

Replication of the nuclear DNA of eukaryotes is markedly sensitive to inhibition of

cytoplasmic protein synthesis. The initiation of DNA replication and chain growth already in progress are both sensitive to protein synthesis (Henshaw, 1980). This sensitivity is observed in the incorporation of ^{3}H-thymidine into liver nuclear DNA in fully fed rats, a diurnal peak occurring 12 hours after the onset of feeding. The marked food dependency of DNA synthesis in the liver is not so apparent in other tissues and is eliminated by feeding a low protein diet (Dallman *et al.* 1974). Inhibition of cell proliferation in response to nutrient deprivation appears to be a protective physiological response rather than simply a direct substrate limitation since such cells will continue through S phase, G2, and M but will become blocked in early G1. The lower tumor incidence in rats on a restricted diet could be related to the poorer survival of tumor cells when nutrients are insufficient (Pardee, 1974).

Prolonged protein deficiency or protein malnutrition results in an increase transcription of rRNA coupled with an increased turnover of rRNA and ribosomes (Kawada *et al.*, 1977; Conde *et al.*, 1980). The restriction of protein in the diet results in an accelerated breakdown of RNA in tissues while absorption of amino acids from the gut halts RNA breakdown (Clark, Naismith and Munro, 1957). There appears to be a relationship between the stability of RNA and the availability of amino acids for protein synthesis. The lower RNA to DNA ratio observed in the liver, kidney, heart, and abdominal skin from chronically dietary-restricted rats is in agreement with the findings of Onishi (1970). Srivastava (1978) studied the metabolism of liver RNA in female Sprague Dawley rats after a period of 13 months during which time they had been maintained on 50% of the diet consumed by *ad libitum* fed, age matched control rats. The lower RNA to DNA ratio observed in the dietary-restricted rats resulted from a rapid degradation of rapidly synthesized RNA. Among the various species of RNAs the tRNA and 4-18S components of the insoluble RNA fraction showed the greatest increase in the rate of synthesis. The free nucleotide content of the plasma increased during undernourishment indicating a rapid breakdown of RNA in the tissues (Srivastava *et al.*, 1972).

With increasing age the rate of DNA synthesis in the fully-fed rats declines and by 6–12 months no significant difference is apparent between control and dietary-restricted rats. The reduction in cell size (protein/DNA ratio) and the reduced content of cellular RNA (RNA/DNA ratio) induced by restricted feeding do not persist beyond one year. The cellular relationship of protein content to RNA content (ribosomal efficiency) is not disturbed at any age in the tissues studied from dietary restricted rats. It is suggested that the reduced synthesis of DNA is a response to a reduced rate of protein synthesis in the liver, kidney, heart and abdominal skin as a result of enhanced RNA degradation in tissues from undernourished rats.

There are many studies which support the concept that dietary restriction which increases lifespan also delays the occurrence of specific programmed events although the overall age-related profile of physiological and pathological changes is retained (Walford *et al.*, 1974; Merry and Holehan, 1979; 1981; Barrows and Kokkonen, 1982). The inhibition of DNA synthesis observed in the present study does not conform with this concept, for the developmental peak of DNA synthesis is not delayed, but inhibited. However, the consequence of this decreased DNA synthesis is to retard the development of tissues as shown by the delay in hepatocyte transformation to higher ploidy states (Enesco and Samborsky, 1983; Gahan *et al.*, 1984).

SUMMARY

Male CFY Sprague-Dawley rats maintained at 50% growth rate of *ad libitum* fed, age-matched control animals showed a 42% extension in maximum lifespan and an increase in the overall doubling time of the rate of mortality from 102 days to 203 days. The slower growth rate of animals on the restricted diet was reflected in lower DNA, RNA, and protein content of liver, kidney and heart with age. DNA synthesis *in vivo* measured by the incorporation of ^{3}H-thymidine was severely inhibited in liver, kidney, heart, and abdominal skin, particularly during the first six months of life. The developmental peak in DNA synthesis observed in liver and kidney of *ad libitum* fed animals before 100 days of age was completely inhibited. No effect of restricted feeding on DNA synthesis in the small intestine was observed; however, DNA synthesis was higher in thymic tissue from the experimental animals. A decrease in RNA content of liver, kidney, and heart preceded any change in protein or DNA in animals subjected to restricted feeding. The capacity for protein synthesis (RNA/DNA ratio) was decreased by restricted feeding and was associated with a reduced protein/DNA ratio indicative of reduced cell size during the first six months of life. The translational activity per ribosome (protein/RNA ratio) was not disturbed by undernutrition in any of the tissues studied.

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NITROGEN BALANCE STUDIES IN AGING RATS

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Abstract—Nitrogen balance studies were conducted to compare the protein requirements of aged (24-month-old) and mature (12-month-old) rats for possible age-related differences. Four diets with protein supplied as amino acid mixtures simulating casein protein at 4.5%, 4.5% plus 1.5% indispensable amino acids of casein, 4.5% plus 1.5% dispensable amino acids of casein or 6.0% casein protein were offered for one week each during a four-week counter-balanced dietary sequence. Lower food and nitrogen intake of aged rats resulted in a lower mean nitrogen balance compared to that of the mature animals. Calculations of the level of N intake necessary to provide for N equilibrium in the aged rats indicated an apparent increase in the actual net need for nitrogen and indispensable amino acids when compared with the needs of the mature animals.

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INTRODUCTION

STUDIES OF possible age-related changes in the protein requirements of aged subjects have provided contradictory results (Watkin, 1964; Swendseid, 1963). However, the importance of determining the amount and direction of such changes regardless of the probable multiplicity of underlying physiological and biochemical mechanisms remains undisputed. Although the population of the United States has shown an increase in the number of individuals aged 65 and older (Tauber, 1972), the recommended levels of intake do not make provision for possible differences in requirements for older individuals (Nat'l Res. Council, 1980). It is assumed that any changes in protein requirement with the onset of senescence in healthy individuals are minimal and that the requirement may be slightly, but insignificantly, lower than the recommended level (Watkin, 1964). However, since the elderly comprise approximately 10% of the national population and incur at least 30% of national health costs, further investigation of the actual requirements of the aged is justified.

If differences between the requirement of the mature and the aged have not been clearly demonstrated, this may, at least in part, be the consequence of the extreme difficulty of obtaining sufficiently comparable subjects, and the inherent problems in the methodology of assessment.

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A number of preliminary studies were carried out to investigate the protein requirements of older rats using the nitrogen balance method (Baldwin, 1978). Based on the results obtained from these preliminary investigations, an experiment was devised to determine whether there was an age-dependent increase in the requirement or a change in the response to nitrogen (N) intake near the maintenance requirement level.

METHODS AND PROCEDURE

Barrier-reared Fischer 344 male rats were used; they were obtained from the National Institute of Aging colony maintained by the Charles River Breeding Laboratories, Wilmington, MA. For the purpose of this study, the term "mature" was assigned to the 12-month-old animals, and the 24-month-old animals were referred to as "aged." Each age group consisted of eight rats. The assignments to treatments and diet offerings were made without regard to initial body weight.

Four test diets (A,B,C,D) were given during each of the five trial weeks in a counter-balanced sequence providing duplicated Latin square blocks for each of the two age groups, thus minimizing the effect of dietary sequencing on the nitrogen responses (Keppel, 1973).

Differences in protein requirement of aged and mature rats were asssessed by the effect of the four test diets (Table 1) on nitrogen balance. N balance was calculated as nitrogen intake minus nitrogen excreted in urine and feces, with integumental losses assumed minimal and essentially equal among all animals. Each experimental diet was given *ad libitum* for seven consecutive days (Table 2). Daily individual N balances were determined for all rats for the last four days of each trial week.

This arrangement of diet presentation and measurement of N balance allowed for the collection of data concerning the effects of age, diet, week and week/day as factors of the protein requirement and provided a total of 32 observations for each diet within each age group.

Diets A (4.5% simulated casein protein, methionine supplemented) and D (6% simulated casein protein, methionine supplemented) were chosen to study the quantitative aspect of the protein requirement of aged vs. mature animals. Diet A had been shown in preliminary tests to provide the minimum protein requirement to maintain older (mature) rats in N equilibrium. Diet D was chosen to provide protein at a level near that requirement, but sufficiently higher to produce a positive N balance as demonstrated in preliminary experiments.

Diets B and C (4.5% simulated casein protein (SC), methionine supplemented, plus 1.5% essential amino acid (EAA) mix, and 4.5% SC, methionine supplemented, plus 1.5% non-essential amino acid (NEAA) mix, respectively) were chosen for evaluation of qualitative aspects of possible differences in the protein requirement of the two age groups.

All diets were analyzed for N content by the Kjeldahl method prior to use. When necessary, they were then adjusted with EAA and/or NEAA mix to provide, as closely as possible, the desired N levels. Animals were housed individually in suspended stainless steel wire bottom metabolism cages. During the entire study, animals were maintained in an isolated controlled environment. Light was provided on a 12 hour off/on cycle. Temperature was maintained at 72–74 F.

TABLE 1. SIMULATED PROTEIN CONTENT OF EXPERIMENTAL DIETS.

Diet	*Amino Acids Simulating Casein*	*Amino Acid Mixtures*	*Basal*[c]
A	4.5	–	94
B	4.5	1.5 EAA[a]	94
C	4.5	1.5 NEAA[b]	94
D	6.0	–	94

[a]Mixture of indispensable (essential) amino acids simulating those found in Casein Protein.

[b]Mixture of dispensable (non-essential) amino acids simulating those found in Casein Protein.

[c]Sucrose 40.0%, Fiber (Solka-floc, Brown Co.) 5.0%, Corn oil 10.0%, Vitamin Mix 0.2% (Bauer and Griminger 1980), Biotin 0.006%, Choline Chloride 0.1%, Salt Mix 4.0% (Draper et al., 1964), $ZnSO_4 \cdot H_2O$ 0.0068%, $CuSO_4$ 0.0038%, Corn starch 34.7, DL methionine, 0.1%. Corn starch added to make up to 100%.

TABLE 2. EXPERIMENTAL DESIGN.

Experimental Animal Pairs[a]	Week on Experiment 1	2	3	4	5
	Experimental Diets[b]				
1,5	A	B	C	D	A
2,6	B	D	A	C	B
3,7	C	A	D	B	C
4,8	D	C	B	A	D

[a]Each of the two pairs on each diet sequence (for example, pair 1 and pair 5) consisted of a mature (12-month old) and an aged (24-month old) rat.

[b]Experimental diets, see Table 1.

Individual food intakes were recorded daily. During the collection period (last four days of each week), urine and feces were collected for each day. While urinary N excretion was measured on a daily basis, feces were pooled for the four-day collection period of each animal and dried to a constant weight prior to N analysis.

RESULTS AND DISCUSSION

Nitrogen balance responses to the 4.5% protein diet (A) were considerably different for the two age groups of rats. The average N balance of the mature animals (9.5 mg/rat/day) on diet A indicates that these animals were close to or in N equilibrium; in contrast, the average N balance of the aged rats was definitely negative (−14.4 mg/rat/day) (Table 3).

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TABLE 3. AVERAGE (±SE) DAILY N INTAKE, N EXCRETION, AND N BALANCE OF MATURE AND AGED RATS ON FOUR DIFFERENT DIETS.

Diet[a]	Age[b]	Nitrogen Intake	Urinary Nitrogen	Nitrogen Balance[c]
		mg	mg	mg
A	M	102 ± 11	58 ± 5	10 ± 5
A	A	79 ± 12	70 ± 7	−14 ± 6
B	M	145 ± 10	65 ± 5	47 ± 5
B	A	116 ± 12	76 ± 8	12 ± 6
C	M	153 ± 12	87 ± 7	31 ± 6
C	A	129 ± 17	100 ± 11	−2 ± 9
D	M	149 ± 15	70 ± 6	45 ± 7
D	A	132 ± 13	87 ± 7	15 ± 8
M (A,B,C,D)		137 ± 14	70 ± 10	33 ± 9
A (A,B,C,D)		114 ± 15	83 ± 9	3 ± 10

[a]Diet A, 4.5% simulated casein; diet B, 4.5% simulated casein plus 1.5% EAA mixture; diet C, 4.5% simulated casein plus 1.5% NEAA mixture; diet D, 6.0% simulated casein.

[b]M = mature (12-month old); A = aged (24-month old) male Fischer 344 rats.

[c]N balance = N intake − (urinary N + fecal N), average of weeks 1–4. Fecal N (not shown) = N intake − (urinary N + N balance).

The difference in N balance is approximately equal and probably directly attributable to the difference in N intake. The lower N balance on diet A observed in the aged rats compared to that of the mature was fairly consistent during each of the four test weeks.

The results indicate that the 4.5% protein diet (diet A) produced a N balance close enough to N equilibrium to be considered as the requirement level for mature rats. However, this diet was insufficient for the maintenance of N equilibrium in the aged rats.

The N balance responses to each of the three 6.0% protein diets (diets B,C,D) of the aged animals during each of the five test weeks indicated in all instances considerably less N retention than those of the mature animals. The average daily N balances of mature animals on diets B,C, and D showed a greater N retention than those seen on diet A and were all strongly positive; the average retentions were 47.3, 31.1 and 44.8 mg/rat/day, respectively, versus 9.5 mg for diet A. The average daily N balances of the aged animals on diets B (12.5 mg/rat/day), C (−1.6 mg/rat/day) and D (15.1 mg/rat/day), were more positive than on diet A (−14.4 mg/rat/day), but were still lower than the N balances observed in the mature animals.

Comparing the combined experimental diets B,C, and D for the two age groups, one obtains N balances of 41.1 mg/rat/day for the mature, and 8.7 mg/rat/day for the aged; this difference of 32.4 mg is slightly more than the difference observed in mean N intake between the two age groups (23.5 mg). Although it was noted that the major factor contributing to the more negative N balance of the aged animals was the lower food intake and, therefore, lower N intake, the urinary excretions of the aged animals were in almost all instances greater than those of the mature.

As previously noted, diets B and D produced more positive N balances than did diet C for both ages (46 and 30 mg vs. 15 mg). Although in both ages the N intake and urinary N were not significantly different among diets, the urinary N per mg of N intake of diet C was higher than that of diets B and D (Table 4). The resultant lower balance on diet C suggests that some difference in N response to the amino acid composition of this diet occurs at both ages. As diet C contains 4.5% simulated casein protein plus 1.5% NEAA, this difference may be the result of a dilution of the effective level of the indispensable amino acids, or of a particular indispensable amino acid.

TABLE 4. RATIO OF URINARY NITROGEN TO NITROGEN INTAKE IN MATURE AND AGED RATS FED THREE APPROXIMATELY ISONITROGENOUS DIETS.

	Diet[b]		
Age[a]	*B*	*C*	*D*
	Average Urinary Nitrogen/ Average Nitrogen Intake		
M	.45	.57	.47
A	.66	.78	.66

[a]See Table 3, footnote [b]
[b]See Table 3, footnote [a]

The apparent digestibility coefficients of the three 6.0% protein diets B, C, and D, which were isocaloric and approximately isonitrogenous, were equal for both age groups, suggesting that the differences noted in N balance were not the result of diet- or age-related differences in the absorption of dietary N.

Since the differences in N balance were seen to be mainly the result of differences in the N consumption by the rats of the two age groups on each of the four diets, a further examination of the differences in response of the mature and aged animals to the test diets was made by a comparison of the amount of N intake necessary to produce equivalent responses. This evaluation was made by calculating a regression line (N balance/N intake) for each age group on each of the four diets (Table 5). The N intake at N equilibrium (NB = 0) was estimated from these lines. As indicated by the R-square value, about two-thirds of all observations of each age-diet group are explained by the regression equation.

Using a computerized statistical analysis system (SAS) to generate analysis of variance and least significant differences, it was determined that the amount of N required for N equilibrium for the aged rats on diet B (105.0 mg) was significantly greater ($p < 0.05$) than the amount required by the mature (78.6 mg) for an equivalent response (Table 5). The N intake necessary for the aged animals to achieve N equilibrium on each of the remaining diets A (98.0 mg), C (136.2 mg), and D (111.2 mg) was larger than the N intake required by the mature on diets A (90.0 mg), C (112.6 mg), and D (83.3 mg). However, these differences did not reach the 0.05 level of significance. The N intake required by the aged to produce N equilibrium on diet C was significantly greater than that on diets A and

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TABLE 5. REGRESSION COEFFICIENTS FOR NITROGEN BALANCE REGRESSED ON NITROGEN INTAKE AND STATISTIC OF FIT OF NITROGEN BALANCE OBSERVED IN MATURE AND AGED RATS ON FOUR DIFFERENT DIETS.[a]

Diet & Age[b]	*NB*[c]	*Intercept*[d]	*Slope*[e]	*R Square*[f]	*NI at NB = 0*[g]
	mg				
A,M	9.1 ± 15.1	−70.4 ± 9.2	0.78 ± 0.1	0.73	90.0 ± 13.0
A,A	−14.4 ± 18.1	−80.3 ± 9.1	0.86 ± 0.1	0.70	98.0 ± 4.2[i]**
B,M	47.2 ± 13.4	−55.8 ± 13.1	0.71 ± 0.1	0.67	78.6 ± 10.0[h]*
B,A	12.5 ± 16.5	−95.5 ± 11.2	0.91 ± 0.1	0.77	105.0 ± 3.3[i]**
C,M	27.6 ± 16.7	−77.7 ± 14.1	0.69 ± 0.1	0.67	112.6 ± 18.6
C,A	−4.4 ± 24.3	−83.1 ± 12.7	0.61 ± 0.1	0.60	136.2 ± 7.2
D,M	43.7 ± 20.0	−55.8 ± 13.6	0.67 ± 0.1	0.66	83.3 ± 18.0
D,A	15.1 ± 21.7	−80.1 ± 14.8	0.72 ± 0.1	0.60	111.2 ± 5.0[i]*

[a]n = 32, 4 (24 hr) N balances of 2 rats of each age group on each diet for weeks 1–4.

[b]Letters denote diet and age (see Table 3, footnotes a and b).

[c]NB = average daily nitrogen balance ($\bar{y}$) ± standard deviation.

[d]Estimated NB at zero nitrogen intake (α) ± standard error.

[e]Slope NB/NI (β) ± standard error

[f]Statistic of fit, percent of observations which are included in estimate.

[g]Average daily NI when NB = 0 ± standard error, calculated from equation of line when NB is regressed against NI, standard error of NI where var = standard error of α or β,

$$(\text{when NB} = 0) = / \frac{\alpha^2}{\beta} \frac{(\text{var } \alpha)^2}{(\alpha)^2} + \frac{(\text{var } \beta)^2}{(\beta)^2} + 2 \frac{(\text{NI}) (\text{var } \beta^2)}{(\alpha) (\beta)}$$

[h]Significant difference between ages on this diet. *$p < 0.05$ **$p < 0.01$

[i]Significantly different from C, A value.

B ($p < 0.01$), and D ($p < 0.05$). While the differences in the mature rats were in the same direction, they were not significant.

The finding that the N intake for aged rats to achieve N equilibrium is more than the amount necessary for the same response in the mature is demonstrated by additional observations. The level of N intake (98.0 to 136.2 mg) required by the aged on all four diets for N equilibrium was higher than that of the mature (78.6 to 112.6 mg). On the three 6.0% protein diets the N intake (mg/kg BW) for N equilibrium of the aged rats was 37% greater than that of the mature. On the 6.0% protein diet with a high indispensable amino acid content (diet B) aged rats required significantly greater N intake (a difference of 83 mg/kg BW) than the mature to maintain N equilibrium.

These results tend to confirm studies conducted in older men which also reported an increase in indispensable amino acid requirements with age (Tuttle *et al.*, 1965). The results obtained on diets C and D in aged animals are also in agreement with the findings that the indispensable amino acids may be used more effectively as the supplemental N source (Tuttle *et al.*, 1965).

The dietary protein level necessary for aged rats to maintain adequate N balance is apparently higher than that of mature animals. This is a result of the lower food intake and, therefore, N intake of the aged animals. However, in addition, comparisons of N balance studies in mature and aged rats have provided evidence for an apparent increase in the actual net needs for N and indispensable amino acids of the aged.

Similarity of results obtained in the aged animals of this study and reported studies in elderly humans indicate that this animal model may be useful in providing experimentally derived information concerning age-related changes in N requirements of aged humans.

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LYMPHOCYTE AGING IN ALLOPHENIC MICE

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Abstract—Allophenic mice are chimeras which are produced from the aggregation of two genotypically distinct embryos. In this study, embryos from the C57BL/6J and A/J strains were used to produce C57BL/6J — A/J allophenic mice. These strains were chosen because of their markedly different lifespans, their different glucose phosphate isomerase (GPI) isozymes, and their different *H-2* haplotypes [C57BL/6J: long-lived, *Gpi*-1^b, *H-2*^{b}; A/J: short-lived, *Gpi*-1^a, *H-2*^{a}]. The mice were bled at two month intervals and the composition of their peripheral blood lymphocytes determined at each point from analysis of GPI isozymes and *H-2* antigens. It was found that the proportion of long-lived (C57BL/6J) lymphocytes tended to increase with age. Moreover, the total lifespan of the allophenic mice was directly related to the percentage of long-lived lymphocytes in their peripheral blood.

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INTRODUCTION

THE IMMUNE system plays an important, perhaps crucial, role in the aging process. It is well documented that aged animals show disturbances in humoral and cellular immune responses, T cell/B cell ratio, ability to respond to lectins, and a myriad of other immune functions (Kay and Makinodan, 1981). It is unknown whether these abnormalities are the cause or the consequence of the aging process. Aberrant immune function in aged animals can lead to death from infectious agents, cancer, or autoimmune disease.

The aging process, leading to a particular aging phenotype, has both genetic and environmental components. In the mouse, a species in which many inbred strains are available, it is clear that different strains have markedly different lifespans (Storer, 1969; Altman and Katz, 1979; Heiniger and Dorey, 1980). Some of the reported differences in lifespan are in strains of animals raised in the same environment, leading to the conclusion that the aging process has a strong genetic component. The definition of "the same environment" is a difficult one to make. There is, however, a system in which cells from two animals of markedly different lifespans may be combined into a single animal, thus creating a "same environment" for both types of cells. This system is the allophenic mouse, first described by Tarkowski in 1961 and Mintz in 1962.

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Allophenic mice are chimeras produced by the aggregation of two embryos early in development. These animals show true mosaicism and no apparent intolerance of the two types of cells for each other. McLaren (1976) has termed allophenic mice primary chimeras because they are potentially chimeric in all their tissues and organs. This distinguishes them from secondary chimeras, such as bone marrow chimeras, which are chimeric in only the hematopoietic system.

Allophenic mice have been used in a number of immunologically interesting studies, including studies on the mechanism of tolerance (McLaren, 1976) and *Ir* (immune response) gene function (Warner *et al.*, 1973; 1976; 1977a; 1978; Gorcyca *et al.*, 1982). It has been reported that the composition of the immune system of allophenic mice changes with age (Stephens *et al.*, 1977; Warner *et al.*, 1977b; Stephens and Warner, 1980), but the mice in these studies were not allowed to get truly "old"; they were all killed to complete the studies by the relatively young age of 8 months. Abnormalities in the immune system of mice do not usually become strikingly apparent until the mice are greater than 16 months old (Kay and Makinodan, 1981).

The purpose of the present study was to combine cells from a short-lived mouse strain and a long-lived mouse strain into a single animal through the creation of allophenic mice. The mice were then bled at two month intervals until death and the composition of their peripheral blood lymphocytes analyzed. The two markers used for the analyses were the enzyme glucose phosphate isomerase (GPI) as an internal marker, and the cell surface antigen *H-2*, as an external marker. The results reported in this paper will show that the lifespan of each allophenic mouse was correlated with the percentage of long-lived lymphocytes in the particular animal.

MATERIALS AND METHODS

Mice

The C57BL/6J and A/J mice were purchased from the Jackson Laboratory, Bar Harbor, ME. The pertinent characteristics of the A/J and C57BL/6J strains are listed in Table 1. It is seen that the mean lifespan of the animals varies from lab to lab, but the A/J mice are always short-lived compared to the C57BL/6J mice. We are currently obtaining lifespan data for these two strains in our own laboratory, but the data are not yet available.

Allophenic mice (C57BL/6J ⟷ A/J) were produced as described previously (Warner *et al.*, 1973; Mintz *et al.*, 1973). Briefly, eight cell embryos were collected from superovulated female mice, the zona pellucida removed with pronase (Calbiochem), the embryos treated with phytohemagglutinin-P (Difco) after which they were bumped together and cultured for about 24 hours in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. The resulting double-sized blastocysts were transferred to pseudopregnant CF1 mice (Charles River) and allowed to

TABLE 1. CHARACTERISTICS OF C57BL/6J AND A/J MICE.

					Mean Lifespan (days)		
Strain	*Coat Color*	Gpi-*1*[1]	H-2-*haplotype*[1]	*Sex*	*Source 1*[1]	*Source 2*[2]	*Source 3*[3]
A/J	albino	a	a	Virgin ♂	662 ± 20	490 ± 18	634
				Virgin ♀	688 ± 18	590 ± 19	694
C57BL/6J	black	b	b	Virgin ♂	827 ± 34	676 ± 20	841
				Virgin ♀	818 ± 21	692 ± 16	789

[1]From Altman and Katz, 1979.
[2]Storer, 1969.
[3]Heininger and Dorey, 1980.

come to term. After weaning, male and female allophenic mice were separated, and same sex littermates kept in the same cage, as virgins, throughout their lives.

Coat Color Estimation

Coat color was estimated by visual inspection at weaning, at 2 months of age and every 2 months thereafter.

Blood Collection and Lymphocyte Isolation

The allophenic mice were bled at 2 months of age and every 2 months thereafter until death. About 0.3 ml of blood was collected from the orbital venous plexus into heparinized hematocrit capillary tubes. The blood was immediately expelled into 0.7 ml of PBS (0.01 M sodium phosphate buffered saline, pH 7.0) containing 0.01% EDTA and 50 IU heparin. The blood was then divided into two 0.5 ml aliquots and layered on 1.0 ml Ficoll-Hypaque (density = 1.088) in each of two 1.5 ml microfuge tubes. The tubes were centrifuged at 10,500 × g for 3 minutes in a Beckman Microfuge 12. The white blood cell layers were collected, resuspended in PBS, and centrifuged at 6,000 × g for 2 minutes in the Microfuge. The pellets were combined, again suspended in PBS and repelleted at 6,000 × g for 1 minute. The remaining red blood cells were removed by suspending the pellets in 250 μl PBS to which 1 ml of ammonium chloride lysis solution (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.1 mM EDTA, pH 7.4) were added. The cells were immediately pelleted again at 6,000 × g for 1 minute and washed twice with PBS under the same conditions. The final pellet was resuspended in 100 μl of PBS which gave a concentration of about 1–3 × 10^6 cells/ml.

At this point 40 μl of the cell suspension were removed for direct use in the cytotoxicity assay for *H-2* antigens, described below. The remainder was freeze-thawed twice to give the lysate that was used for isozyme analysis, as described below.

Assay for Glucose Phosphate Isomerase (GPI)

Analysis of GPI isozymes was as previously described (Balinsky *et al.*, 1983). The cell lysates were run on Cellogel electrophoresis strips, the enzyme bands stained, and then the Cellogel strips were scanned in an LKB densitometer. All assays were run at least in duplicate. The total areas under the curves were calculated and the percentage of the two isozymes calculated from the total.

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Assay for H-2 Antigens

Antisera for the detection of *H-2* antigens were prepared by reciprocal immunization of the C57BL/6J and A/J mice with spleen cells according to Batchelor (1973). For convenience, these antisera are designated anti-$H\text{-}2^a$ and anti-$H\text{-}2^b$, because *H-2* is the immunodominant antigen on spleen cells. The antisera may contain antibodies to other cell surface antigens in addition to *H-2*, but this is inconsequential to these studies. For the experiments described in this paper, in which the proportion of cells of the A/J vs. the C57BL/6J lineage is being detected in allophenic mice, it is advantageous to use antisera made in reciprocal strains to eliminate any possible cross-reactivity of the antisera (Warner *et al.*, 1977c). The cytotoxicity assays were as described previously (Warner *et al.*, 1977c). A minimum of 200 cells were scored for viability by trypan blue dye exclusion. Each experiment and the controls were run in duplicate, and the results normalized to controls containing only A/J or C57BL/6J cells.

RESULTS

A total of 23 C57BL/6J ⟶ A/J allophenic mice, from 10 different litters, were produced for this study. Data on the first litter is now complete, because all the animals have died. The pertinent sex and lifespan data of this litter are shown in Table 2. The animals

TABLE 2. CHARACTERISTICS OF C57BL/6J ⟶ A/J ALLOPHENIC MICE.

Mouse Designation	*Sex*	*Lifespan (days)*
AG-0011-1	♂	483
AG-0012-1	♂	645
AG-0013-1	♂	649
AG-0014-1	♂	790

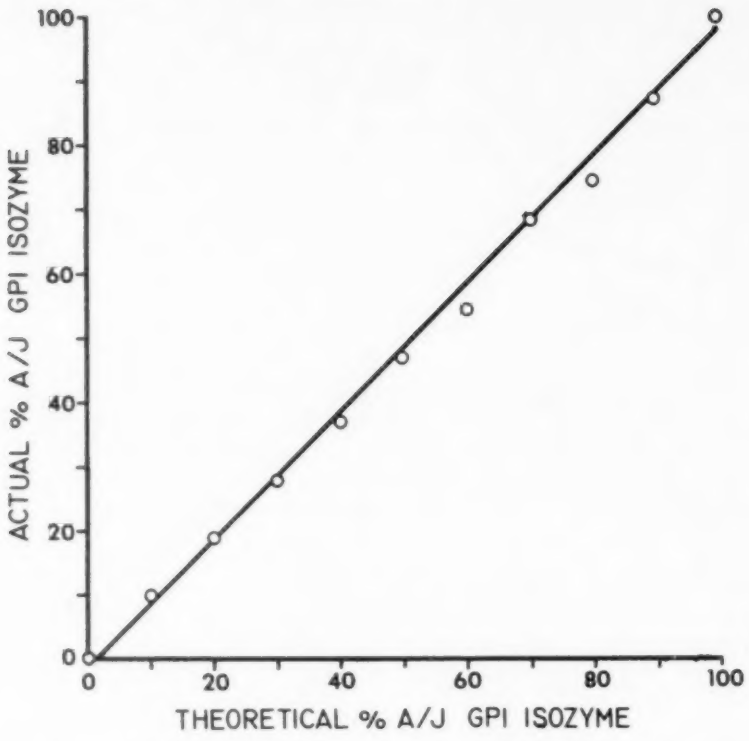

FIG. 1. The percentage of *Gpi*-1^a isozyme of the total in known mixtures of C57BL/6J and A/J spleen lymphocytes. The least squares linear regression line is shown (y = 0.98x − 1.00, r = 0.99).

were visually examined for coat color and bled at 2 month intervals throughout their lives. The isolated peripheral blood lymphocytes were tested for GPI isozyme composition and *H-2* antigen composition. To evaluate the methods used for detection of GPI isozymes and *H-2* antigens, experiments were performed in which known quantities of lymphocytes were mixed together and then analyzed. Figure 1 shows the results of mixing experiments for GPI analysis. These results have been corrected to reflect the fact that on a per cell basis, A/J lymphocytes often have more enzyme activity than C57BL/6J lymphocytes (Warner and Briggs, unpublished). Figure 2 shows the results of mixing experiments for cell surface antigen analysis. No correction was necessary for the *H-2* antigen analysis because the cytotoxicity assay was done at an antiserum concentration which would kill all the cells of one type or the other. The *H-2* analysis is therefore a direct reflection of the proportion of the two types of cells in the peripheral blood of the allophenic mice.

The results of the analysis of coat color, glucose phosphate isomerase isozymes, and *H-2* antigens, as a function of age, are shown in Figures 3, 4, and 5, respectively.

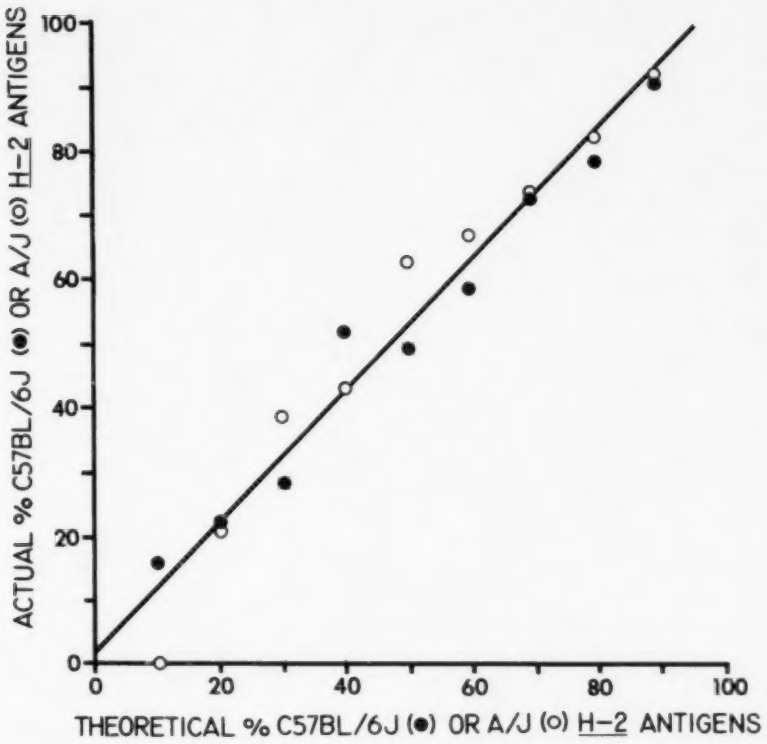

FIG. 2. The percentage of *H-2* antigen expressing cells in known mixtures of C57BL/6J and A/J spleen lymphocytes. Two independent determinations were made using either anti-$H\text{-}2^a$ (○) or anti-$H\text{-}2^b$ (●) antisera. The least squares linear regression line is shown ($y = 0.99x + 2.76$, $r = 0.98$).

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DISCUSSION

This paper is the first report of the use of allophenic mice for aging research. The mice were allowed to live out their full lifespans, and were monitored for coat color and the composition of their peripheral blood lymphocytes at two month intervals. The C57BL/6J and A/J strains were chosen for these studies because of their markedly different lifespans and their different *H-2* and GPI isozyme genotypes.

The coat color of the three mice with marked chimerism at two months of age (AG-0011-1, AG-0012-1, and AG-0013-1), showed a steady increase in albino hair with age (Figure 3). (Inbred C57BL/6J mice remain black throughout their lives.) A study by Gearhart and Oster-Granite (1981) has shown that BALB/cByJ ⟷ C57BL/6J allophenic mice also get progressively whiter as they age, even though the animals were observed only up to 10 months of age. This infers that there is a progressive loss of black melanocytes as allophenic mice age.

The composition of the peripheral blood lymphocytes was evaluated as a function of

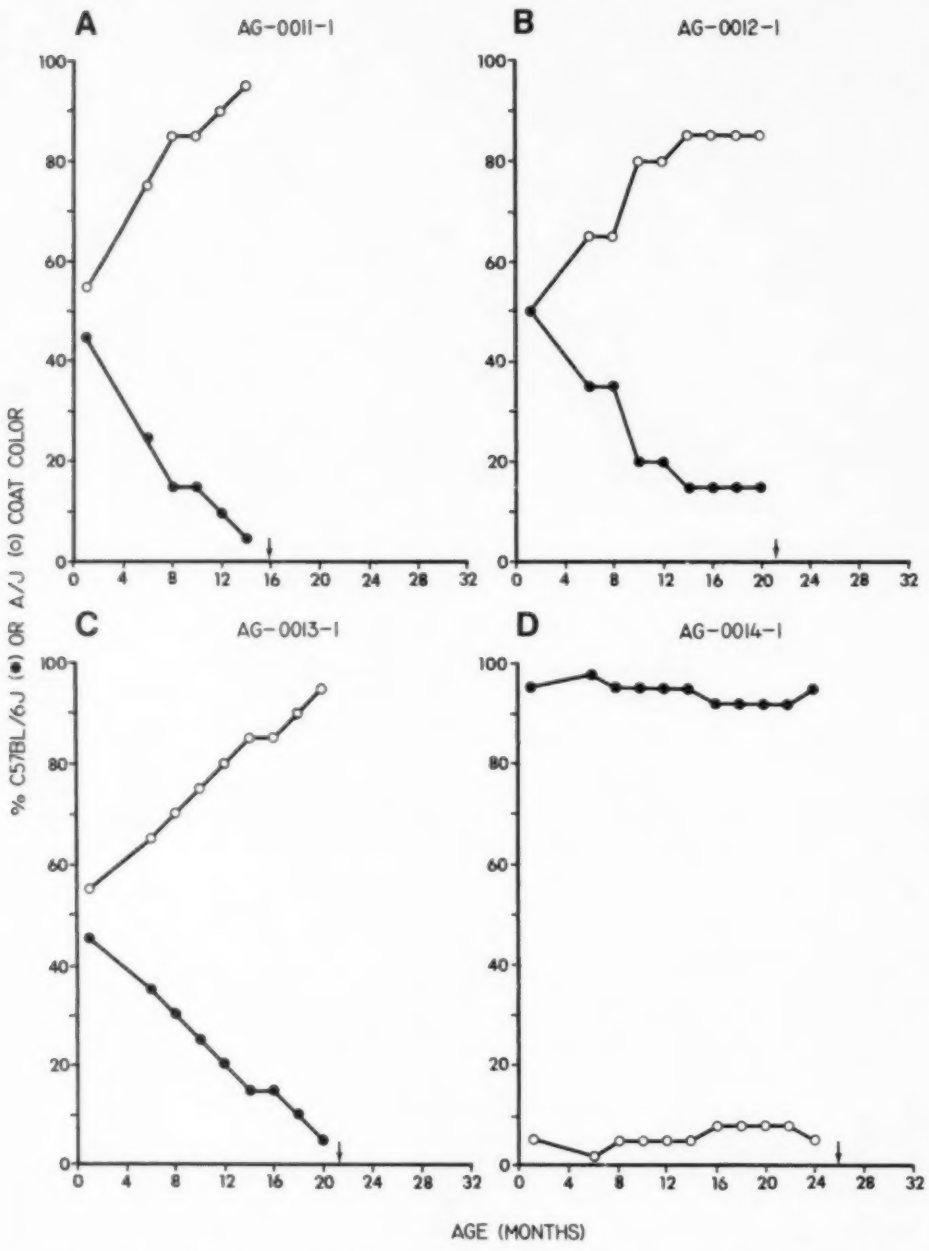

FIG. 3. Change in coat color composition of allophenic mice with age. The percentage of albino (○) or black (●) hair, and the age at death (↓) are shown.

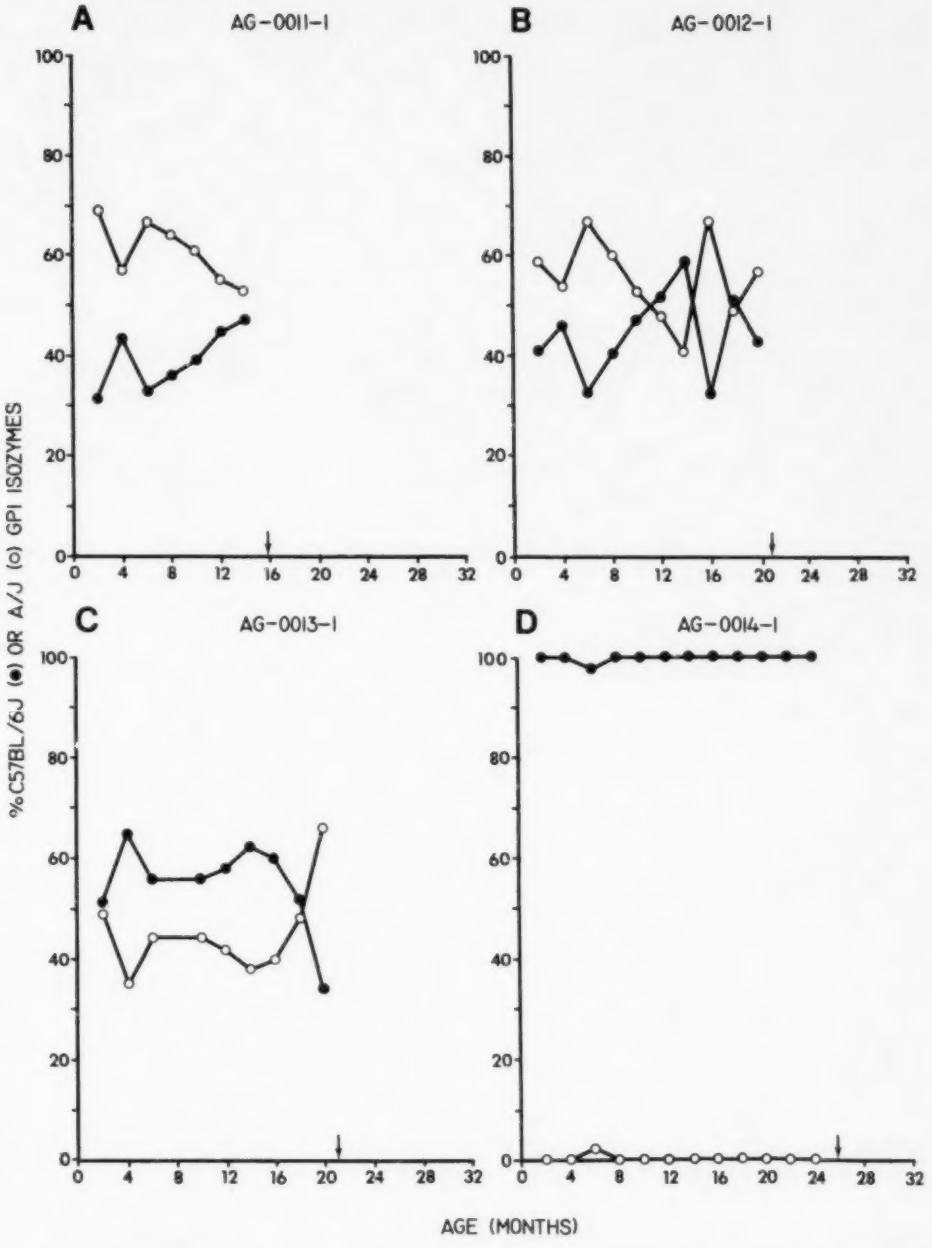

FIG. 4. Change in GPI isozyme composition of peripheral blood lymphocytes of allophenic mice with age. The percentage of *Gpi*-1^a (○) or *Gpi*-1^b (●) isozymes, and the age at death (↓) are shown.

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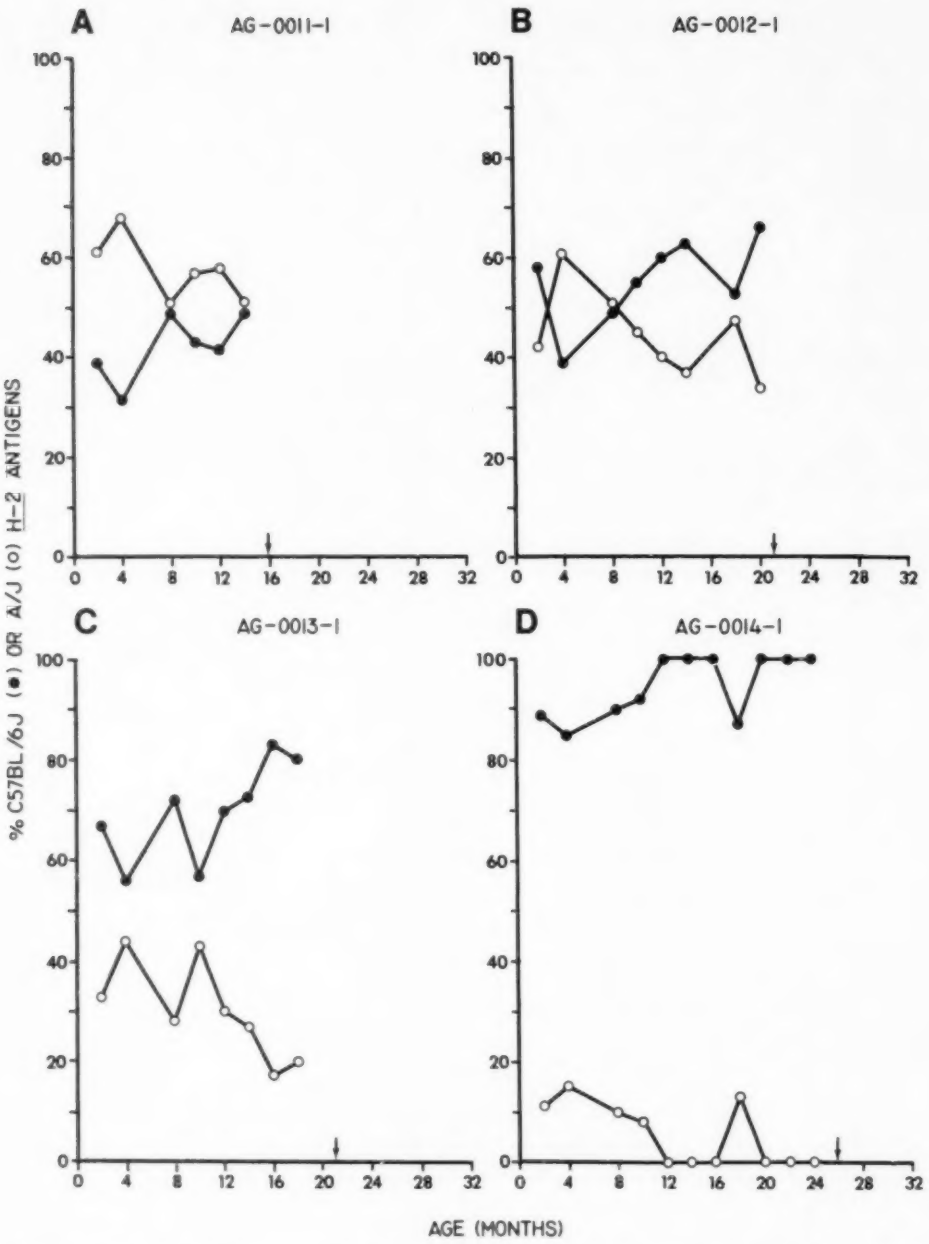

FIG. 5. Change in *H-2* antigen composition of peripheral blood lymphocytes of allophenic mice with age. The percentage of *H-2*^{a} (○) or *H-2*^{b} (●) cells, and the age at death (↓) are shown.

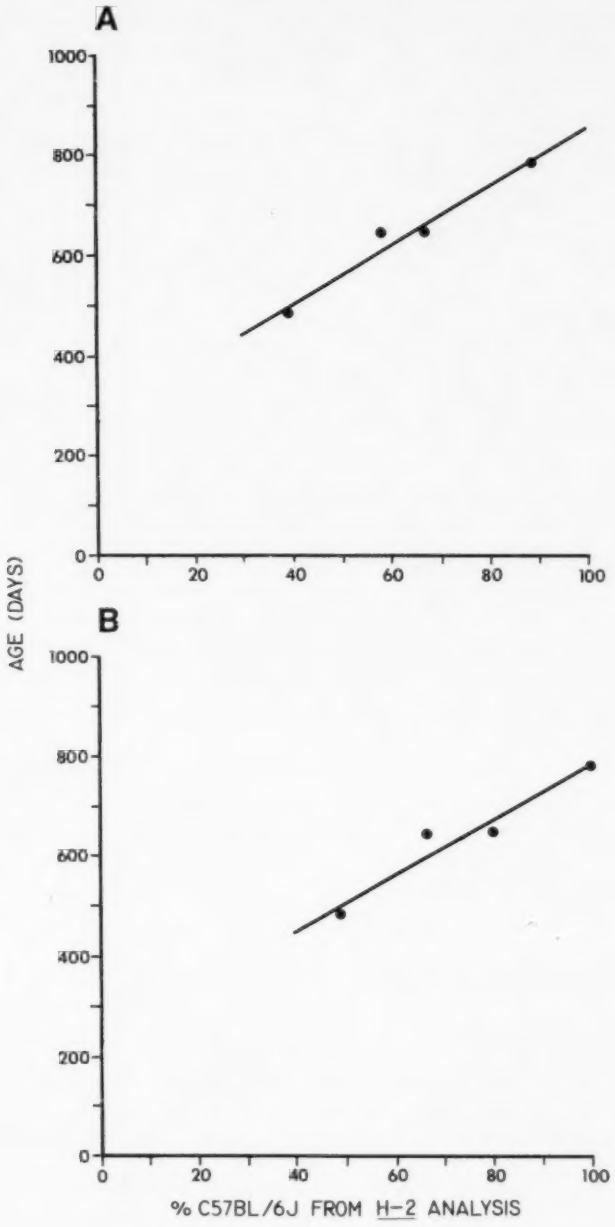

FIG. 6. Correlation of age at death with % C57BL/6J peripheral blood lymphocytes. Data in panel A are from *H-2* analysis at 2 months of age; data in panel B are from *H-2* analysis just prior to death. The equation for the least squares linear regression line in panel A is $y = 5.94x + 266$, $r = 0.98$; the equation for the least squares linear regression line in panel B is $y = 5.60x + 229$, $r = 0.96$.

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age. Both GPI isozyme analysis (Figure 4) and *H-2* antigen analysis (Figure 5) indicate that the relative proportions of the two types of lymphocytes, C57BL/6J and A/J, were unstable as a function of age. This instability has been termed "chimeric drift" (Stephens *et al.*, 1977) and has been observed previously for both red blood cells (Mintz and Palm, 1969; West, 1975, Stephens *et al.*, 1977; Warner *et al.*, 1977b; Stephens and Warner, 1980) and white blood cells (Stephens *et al.*, 1977; Warner *et al.*, 1977b; Stephens and Warner, 1980) in allophenic mice. A recent paper by Behringer *et al.* (1984) suggests that chimeric drift only occurs in mice which differ in *H-2* haplotype. The mice in this study do, of course, differ in their *H-2* haplotypes. The most interesting trend which we have observed in this study is the tendency of the proportion of C57BL/6J (long-lived) peripheral blood lymphocytes to increase with age. This is clearest in Figure 5 (*H-2* analysis) which, as discussed previously, is the most accurate reflection of the true proportion of the two types of parental cells in the peripheral blood of the allophenic mice. There is no correlation of changes in the peripheral blood composition to changes in coat color (compare Figures 3 and 5).

In Figure 6, the age at which each of the allophenic animals died is plotted as a function of the percentage of long-lived (C57BL/6J) cells at the initial time point tested (2 months) and at the last time point tested before death. The percentage of C57BL/6J cells is based on the data in Figure 5. The least squares linear regression lines have been plotted in Figure 6. The line in panel A (2 month data) has a correlation coefficient, $r = 0.98$, and the line in panel B (last point before death) has a correlation coefficient, $r = 0.96$. In fact, similar correlations exist for all the ages tested. Thus, at two months of age it could be predicted which mouse would live the longest and which would live the shortest, based on the relative contribution of short-lived and long-lived cells to the peripheral blood lymphocyte population.

In summary, analysis of a litter of four C57BL/6J ⟷ A/J allophenic mice has shown that as they aged their coat color got whiter, the relative proportion of C57BL/6J (long-lived) peripheral blood lymphocytes increased, and their ultimate lifespan was a function of the proportion of long-lived lymphocytes in their blood. The allophenic mouse should be a useful tool for future studies on the aging of genotypically distinct cells in a common environment.

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CHANGES IN 5′ NUCLEOTIDASE AND ADENOSINE DEAMINASE DISTRIBUTION ACROSS THE RAT LEFT VENTRICLE WALL DURING GROWTH AND AGING

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Abstract—The concentrations of the adenosine-generating enzyme 5′-Nucleotidase (5′-N) and of the adenosine-degrading enzyme adenosine deaminase (ADA) in the rat left ventricle change as a function of the age of the animal. The enzyme distribution across the left ventricle wall is non-uniform in adult or old rats (in the case of 5′-N) or in all age-groups (in the case of ADA). In the oldest rats, 5′-N activity exhibited a significant increase in the midmyocardium and in the inner myocardial layers as compared with the young adult controls.

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INTRODUCTION

It is generally recognized that aging is a significant factor in myocardial performance and disease and that heart disease is common during old age. So far, attention has been focused on age-related morphological changes in myocardial cells (Travis *et al.*, 1972) and in the coronary arteries (Roberts, 1975). Nothing is known about the effects of aging on the biochemical mechanisms that control the microcirculatory blood flow to the different myocardial layers according to the local needs of oxygen.

In this report, we show that heart growth and age have a remarkable effect on the total activities and on the transmural distribution of 5′-Nucleotidase (5′-N) and of adenosine deaminase (ADA) in the rat left ventricle wall. These two enzyme activities may have an important role in the formation and in the catabolism of adenosine (Arch *et al.*, 1978; Schrader *et al.*, 1981), a powerful vasodilator that is possibly involved in the regulation of vascular resistance (Berne, 1963).

MATERIALS AND METHODS

Albino rats of Sprague-Dawley strain respectively 2, 4, 8 or 52-weeks-old were used. After a 12 h fasting period, rats were anesthetized by an intraperitoneal injection of nembutal (5 mg/100 g b.w.) and killed by the rapid injection of 1.0 ml cold 150 mM KCl. The arrested, fully relaxed hearts were rapidly removed and rinsed in ice-cold St. Thomas' cardioplegic solution (Jynge *et al.*, 1981) and a piece of the left ventricle was excised and

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frozen between two aluminum blocks at −30 °C without any compression and cut in 100 μm thick sections in a cryostat at −25 °C as described (De Tata *et al.*, 1983a). Two adjoining sections were combined and homogenized in 1.0 ml 0.1 M Tris-Cl buffer pH 7.4 in a Potter-Elvehjem apparatus (Thomas, Philadelphia, PA).

5′-N was assayed according to Ipata (1967) on the whole homogenate (more than 90% of the enzyme activity is recovered in the particolate fraction under these experimental conditions, De Tata *et al.*, 1983b): 0.25 ml total homogenate were added to 1.75 ml of assay mixture containing (final concentrations) 0.07 mM 5′-AMP; 0.1 M Tris-Cl buffer pH 7.4 and 20 μg/ml adenosine deaminase (type I, SIGMA Chem. Co., St. Louis, MO). Temperature was 37 °C. After 0, 10 and 20 min incubation, aliquots of 0.6 ml of the mixture were injected into 0.6 ml 1.2 N $HClO_4$. Samples were centrifuged and their optical density was measured in a Beckman DU-8 spectrophotometer at 265 nm. Interference in the assay of non-specific phosphatase can be ruled out since no adenosine formation could be detected when 5′-AMP was substituted with an equivalent amount of 3′-AMP.

ADA was assayed spectrophotometrically on the 15,000 g × 20 min supernatant of the homogenate (in these conditions, more than 95% of the heart muscle activity was found to be recovered in the 105,000 g supernatant). 0.05 ml supernatant were added to 1.0 ml of an assay mixture containing (final concentrations) 0.07 mM adenosine in 0.1 M Tris-Cl buffer pH 7.4 and the changes in optical density at 265 nm were recorded (Kalckar, 1947). Temperature was 37 °C.

All data are given as units (μmoles of consumed substrate/min per mg of protein in the total homogenate). Protein determinations were performed by the Lowry et al. procedure (1951) using bovine serum albumin as standard. All of the chemicals used were of analytical grade.

RESULTS

Table 1 shows the changes in heart 5′-N and ADA activities in the growing and in the ageing rat. Both enzyme activities are at their highest levels in the very young rats, and then decrease to a similar extent (the ratio 5′-N/ADA remains almost constant). However, in the oldest age-group, 5′-N activity (but not the ADA activity) exhibits a significant increase above the young adult levels.

Figures 1 and 2 show that both 5′-N and ADA activities exhibit a nonuniform distribution pattern across the left ventricle wall: in all age-groups 5′-N activity are higher in the subepicardial and in the subendocardial layers than in the mid-myocardium (differences are significant only in young adult and in old rats); the ADA activity exhibits a reversed distribution pattern. These data are in accord with previous observations (De Tata *et al.*, 1983b).

The 5′-N and ADA activities exhibit a significant change with age, which may not be similar in all myocardial layers, so that typical patterns of enzyme distribution become apparent. Most interesting, in the oldest rats the distribution of 5′-N activity parallels that one of the young adult controls only in the outer myocardial layers. In the mid-myocardium and inner myocardial layers the 5′-N activity is significantly higher than in younger rats. This rise in 5′-N may account for the significant increase in the enzyme activity reported in Table 1.

TABLE 1. 5′-NUCLEOTIDASE (5′-N) AND ADENOSINE DEAMINASE (ADA) ACTIVITIES IN THE LEFT VENTRICLE OF RATS OF DIFFERENT AGE.

Age (weeks)	*2*	*4*	*8*	*52*
5′-N	18.0 ± .9	18.8 ± .4	11.7 ± .6	14.1 ± .5
ADA	9.8 ± .6	9.2 ± .3	6.4 ± .2	6.4 ± .1

Means of 6 cases ± S.E. are given. Enzyme activities are given as mU/mg protein in the whole homogenate (proteins were assayed according to Lowry et al. (1951) using bovine albumin as the standard. Statistical analysis (F test) showed that both enzyme activities decrease significantly with ageing ($p < 0.01$) with different patterns (interaction is significant, $p < 0.05$).

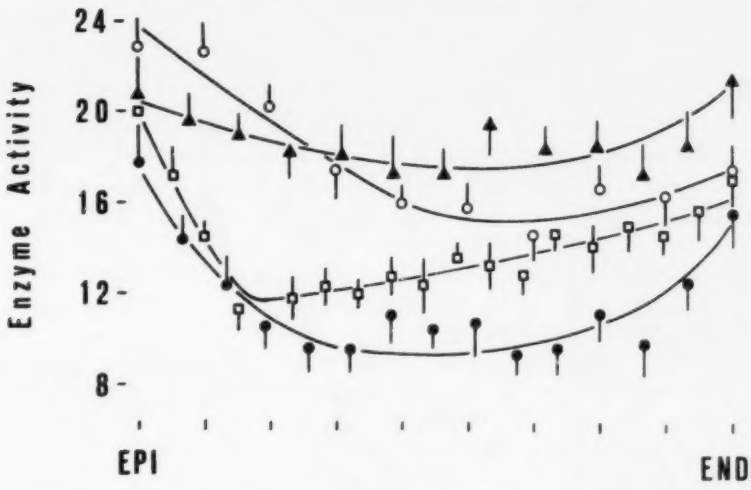

FIG. 1. Transmural distribution of 5'-Nucleotidase activity in the left ventricle of rats of different age. Means of 5 cases are given. Vertical bars represent 1 or 2 × S.E.M. On the abscissa: relative location of tissue in a normalized (100% thick) myocardial wall (the true thickness of the wall in the different age-groups of rats can be calculated from the respective number of fractions × 0.2 mm). On the ordinate, the enzyme activity as mU/mg protein in the whole homogenate. The following symbols are used: (○) 2-weeks-old rats; (▲) 4-weeks-old rats; (●) 8-weeks-old rats; (□) 52-weeks-old rats.

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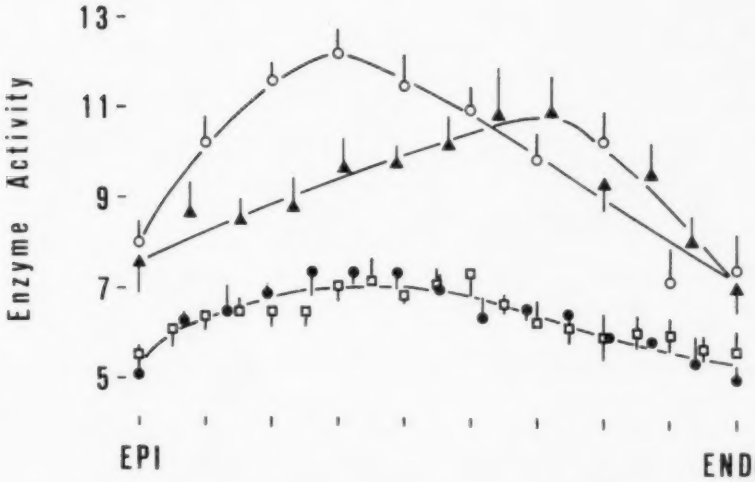

FIG. 2. Transmural distribution of adenosine deaminase activity in the left ventricle of rats of different age. Means of 5 cases are given. Vertical bars represent 1 or 2 × S.E.M. On the abscissa: relative location of tissue in a normalized (100% thick) myocardial wall (the true thickness can be calculated as described in Fig. 1). On the ordinate, the enzyme activity as mU/mg protein in the whole homogenate. Symbols: see Fig. 1, the legend.

DISCUSSION

Our data show that the activity of an adenosine-generating enzyme in the rat left ventricle wall changes with the age of the animal. 5′-N, a membrane bound enzyme, is known to be present in different heart cells, i.e. in endothelial and in myocardial cells (Rubio *et al.*, 1973). In these latter cells, the enzyme activity is associated with the T tubules, the sarcolemma, the intercalated discs and the flattened sarcoplasmic reticulum (Rubio *et al.*, 1973; Nakatsu *et al.*, 1972 and unpublished). Thus, most likely the myocardial enzyme activity increases with cell surface and not with cell volume growth. Perhaps, this factor may account for the relative decrease with age of this enzyme activity in the growing heart. In any case, we should comment that this speculation is not an appropriate one to understand the mechanism of the 5′-N increase in the oldest rats. Perhaps, this latter increase is related to the effect of local factors, since it can be shown in the inner myocardial region only. It is inviting to speculate that the factors may be the nutrition of these tissue layers in the aging heart. In fact, the inner layers of myocardium may be more likely to become hypoxic (see e.g. Hoffman, 1981). In any case, the comment is appropriate that these changes in enzyme activity may have different cellular basis: e.g. they may result from an increase in the endothelial cell number or from a relative increase in the surface/volume ratio in the myocardial cells of these areas.

Several studies support the hypothesis of different nutrition and metabolism of the subendocardial region of the heart (Kirk *et al.*, 1964; Lundsgaard-Hansen *et al.*, 1967; Takenaka *et al.*, 1974). We could observe transmural differences in glycogen turnover and in the transmural distribution of glycogen metabolizing enzymes (De Tata *et al.*, 1983a). Differences in the rate of glycogen turnover may reflect differences in the muscle work load (Villa Moruzzi *et al.*, 1981). Very recently, we showed that two of the enzyme activities of adenosine metabolism (5′-N and ADA) are not distributed uniformly across the myocardial wall (De Tata *et al.*, 1983b). The activity of 5′-N was the highest both in the subepicardial and in the subendocardial layers of the myocardium, where the ADA activity is at its minima. Here we report a very similar distribution in different age-groups of rats. We may mention that we observed the same pattern of enzyme activity distribution in the left ventricle wall of different animal species (including dog, ox and pig) (unpublished). On the contrary, a uniform transmural distribution of 5′-N was observed in the right ventricle wall of the normal rat heart (unpublished). Incidentally, in the normal dog left ventricle the levels of adenosine may be higher in the subepicardial and in the subendocardial muscle layers than in the mid-myocardium (Foley *et al.*, 1979, Figure 1). Perhaps, the adenosine-generating enzyme machinery was given a nonuniform location in the left ventricle wall in order to provide larger vasodilatory capabilities in the subepicardial and in the subendocardial myocardium, where the work load may be the heaviest (Stein *et al.*, 1980a; Stein *et al.*, 1980b). In this perspective, the changes in the 5′-N transmural distribution in the oldest rats may afford a better protection against hypoxia in the mid and inner myocardial regions.

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THE EFFECT OF AGE ON THE CONTROL OF WATER CONSERVATION IN THE LABORATORY MOUSE–METABOLIC STUDIES

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Abstract—Age-related changes in the intake of food and water, and the output of faeces and urine were investigated in C57BL/Icrfa[t] mice of 6 and 24 months of age. Animals were singly housed in a metabolic cage for a period of 30 days. 14 days were allowed for acclimatization before the animals were dehydrated for 24 hours. 10 days of rehydration were allowed prior to a hyperosmotic challenge with 3% sodium chloride in the drinking water. The animals were then observed for 5 more days of rehydration. Urine was collected and analysed with regard to sodium, potassium, urea and vasopressin output/24 hours (/100g body weight), and the osmotic pressure of the urine was determined. Data were analysed by a 2 factor analysis of variance with repeated measures on one factor. Significant changes were detected in the control of body weight, potassium, sodium and urea outputs. No age-differences were detected in the intake of food or water, the output of faeces or urine, the urine osmotic pressure or the excretion of vasopressin. However, significant changes in these variables were detected in both age groups on the days of physiological challenge. The conclusion drawn is that in the mouse strain studied, and for the period of the lifespan investigated, there is no age related defect in the secretion of vasopressin. However, there are trends in the data suggesting a decreased responsiveness of the kidney with age.

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INTRODUCTION

THERE HAVE been many studies of the hypothalamo-neurohypophyseal-renal axis in both laboratory rodents and humans (Davies, 1983). For many years it was accepted that aging was accompanied by a mild form of pituitary diabetes insipidus in humans (Findley, 1949), and studies on the laboratory rat confirmed this (Friedman and Friedman, 1957, 1963, 1964; Friedman *et al.* 1960, 1967; Friedman *et al.* 1965). To summarize briefly, it was shown that the main age-defect was a redistribution of water and electrolytes which could be "corrected" by chronic treatment with neurohypophyseal extracts (Friedman *et al.* 1965, Friedman *et al.* 1967).

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(*Received* 25 *June* 1984)

Some qualitative morphological studies of the hypothalamo-neurohypophyseal region have confirmed that age-changes do occur and have suggested that there may be defects in the ability of this region to synthesize and release AVP (references in Davies, 1983). However, quantitative investigations have shown that there are few age-related changes detectable in this region, even under conditions of osmotic challenge (Davies *et al.* 1983, Davies *et al.* 1984).

Metabolic studies of this axis have been relatively rare since Friedman's early papers, but several recent investigations have produced ostensibly different results (Fliers and Swaab, 1983; Sladek *et al.* 1981; Turkington and Everitt, 1976). In view of these findings, and prompted by our investigations of the hypothalamo-neurohypophyseal system we carried out a detailed investigation of the water intake and urine output of the experimental mouse strain used in our exhaustive quantitative morphological studies.

MATERIALS AND METHODS

Animals

Male C57BL/Icrfa[t] mice were group-housed under standard conditions in a colony of aging animals (Davies and Fotheringham, 1980). Mice aged 6 or 24 months were confined to metabolic cages as described below.

Metabolic Cages

For this experiment, single animals were taken at random and confined to glass metabolic cages (Jencons Ltd., London, UK) for a period of 30 days. Slight modifications had to be made to the feeding apparatus. The standard food-hopper was replaced with a straight glass tube which was of an internal diameter similar to that of the standard food-pellets. The tube contained several food pellets and a spring with a rubber bung sealing the end. The food was restrained by a glass hook at the end of the tube and held by the force of the spring behind, this modification circumvented difficulties that old animals had in eating from the standard food-hopper. Waste food particles fell into a container set beneath the food tube so that there was limited contamination of the urine and faeces.

Animals were put into the cages at approximately 09.00 hours and the following variables were investigated every 24 hours—animal weight, food- and water-intake, and faeces- and urine-output (weight and volume respectively). The urine was subsequently analyzed with regard to osmotic pressure, and sodium, potassium and arginine vasopressin (AVP) output/24 hour period. A microscopical study was made of the urinary deposits, and a chemical analysis of urinary protein, blood, ketones, glucose and pH using LABSTIX* (Miles Laboratories, Slough, UK).

The animals were allowed to acclimatize to the cage conditions (i.e. from group- to isolated-housing) over a period of 14 days prior to 24 hours dehydration by water removal. This was followed by 10 days rehydration prior to 24 hours osmotic challenge by the administration of 3% sodium chloride in the drinking water. A further 5 days rehydration was allowed after this challenge.

Urine Analysis

Electrolytes: The urine electrolytes were determined by atomic absorption spectroscopy (Pye Unicam Ltd.).
Urea: The urine urea was determined by a colorimetric technique based on the Berthelot reaction (Boehringer Corporation [London] Ltd.).
Osmotic pressure: The osmotic pressure was determined by the freezing point depression technique (Advanced Instruments Ltd.).
Arginine vasopressin: The urine AVP was investigated using an RIA developed in this laboratory (Kirkland *et al.* 1983). Dilutions of mouse urine were parallel to the standard curve of AVP.

Statistics

The experimental data were examined using a two-factor analysis of variance (ANOVAR) with repeated measures on one factor. The two factors were age (young, old) and time (days in the experimental period), the second factor being the repeated measure. Each variable was analysed separately using the BMDP(2V) computer package (Dixon and Brown, 1979). The ANOVARs tested for age group and time effects and for an age/time interaction, as well as providing pooled estimates for the between-animals and within-animals standard deviations

(s.d.). A significant age/time interaction term signified that the changes within the two age groups over the experimental period were not equal and these differences were examined in detail using a multiple comparison test (Tukey, 1962). Missing observations were handled by the method of dummy covariates (Bartlett, 1937). Significance was set at 5% throughout.

RESULTS

As the observations for each variable did not vary significantly over the five days prior to each challenge, the data were split into two subsets for analysis. The first subset covered days 11 to 21 inclusive, while the second covered days 20 to 30 inclusive. Thus, the first analysis related to the effects of dehydration and subsequent rehydration and the second to the effects of an acute osmotic challenge and subsequent rehydration. All the data were adjusted to 100g body weight.

Body weight

The age/time interaction was significant in the dehydration phase ($p = 0.002$) and extremely close to significance in the osmotic loading phase ($p = 0.060$). Thus, although both challenges affected the control of body weight, the changes were significantly greater in the old than in the young animals (Figure 1).

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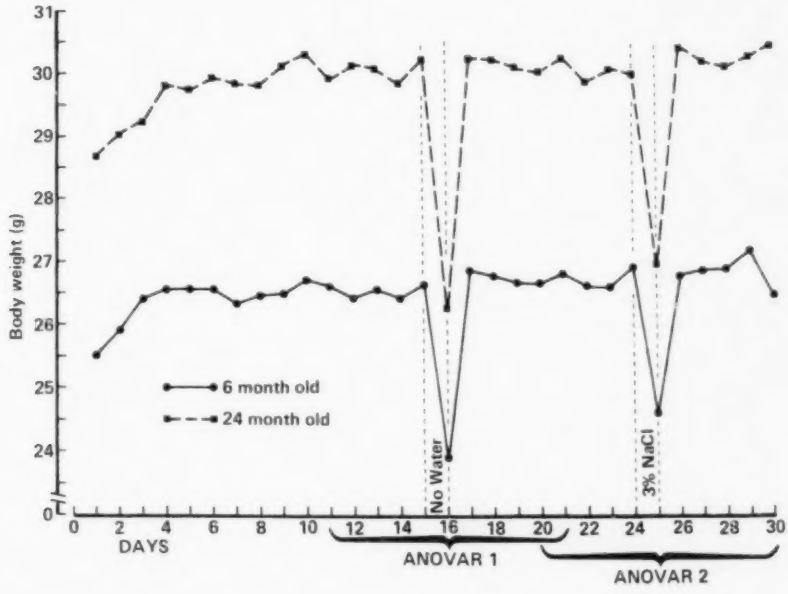

FIG. 1. *Body weight.* Data for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	14.480, $p = 0.003$	F(1,10)	12.995, $p = 0.004$
Time effect:	F(10,110)	96.419, $p < 0.001$	F(10,110)	29.983, $p < 0.001$
Age/time interact.:	F(10,110)	3.072, $p = 0.002$	F(10,110)	1.848, $p = 0.060$

Food intake

Over the dehydration phase, the mean daily intake of food for the young and old animals was 17.3g and 17.5g respectively (between-animals s.d. = 3.7g, $p = 0.836$). On the day of water deprivation, a significant reduction in food intake occurred ($p < 0.001$) which was equal in both groups of animals ($p = 0.463$).

Over the period of osmotic challenge, the mean daily intake of food for the young mice was 16.5g and for the old mice was 16.0g (between-animals s.d. = 5.4g, $p = 0.625$). A significant reduction in food intake was observed on the day of the challenge ($p < 0.001$), the change being equal in the two animal groups ($p = 0.458$).

Water intake

A significant increase in water intake was observed during the day immediately following both dehydration and osmotic loading ($p < 0.001$ for both challenges). As with food intake, there were no age group differences ($p = 0.909$ and $p = 0.883$), the changes being the same for the young and old animals in both experimental phases ($p = 0.550$ and $p = 0.138$) (Figure 2).

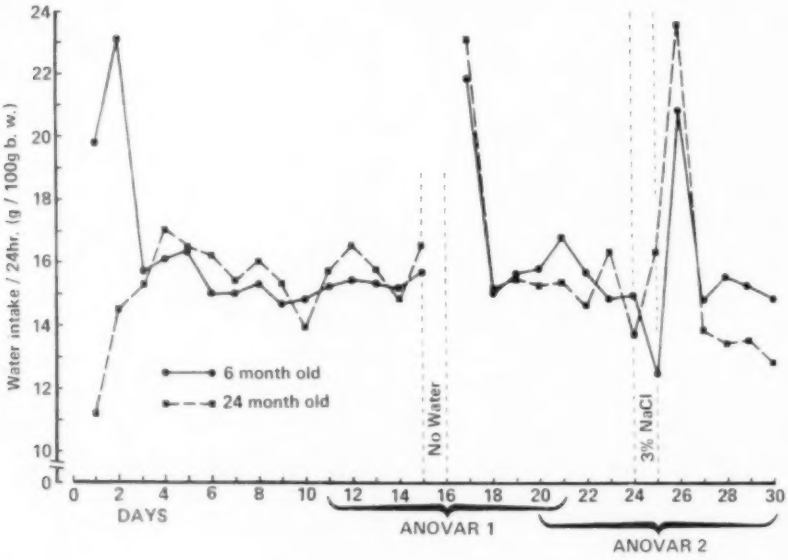

FIG. 2. *Water intake/24 hours (g/100g body weight).* Data for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	0.014, $p = 0.909$	F(1,10)	0.023, $p = 0.883$
Time effect:	F(10,110)	156.421, $p < 0.001$	F(10,110)	7.771, $p < 0.001$
Age/time interact.:	F(10,110)	0.885, $p = 0.550$	F(10,110)	1.529, $p = 0.138$

Faeces output

During the dehydration phase, the mean faeces daily output of the younger mice was 8.3g compared with 9.6g for the old group (between-animals s.d. = 4.2g). On the day of dehydration, faeces output was reduced in the young group but not in the older animals and this difference was significant ($p = 0.036$). The osmotic challenge phase produced mean daily faeces outputs of 8.1g and 8.7g for the young and old mice respectively (within-animals s.d. = 4.8g, $p = 0.447$). Faeces output was significantly reduced on the day of loading ($p = 0.007$) by the same amount in both groups of animals ($p = 0.561$).

Urine output

During the dehydration phase of the experiment, urine output appeared to fall on the day of challenge in the young animals while remaining stable in the old group. The difference, however, was not statistically significant ($p = 0.120$). Urine output increased on osmotic loading. The change in the old group was significantly greater than that in the young group ($p = 0.014$) (Figure 3).

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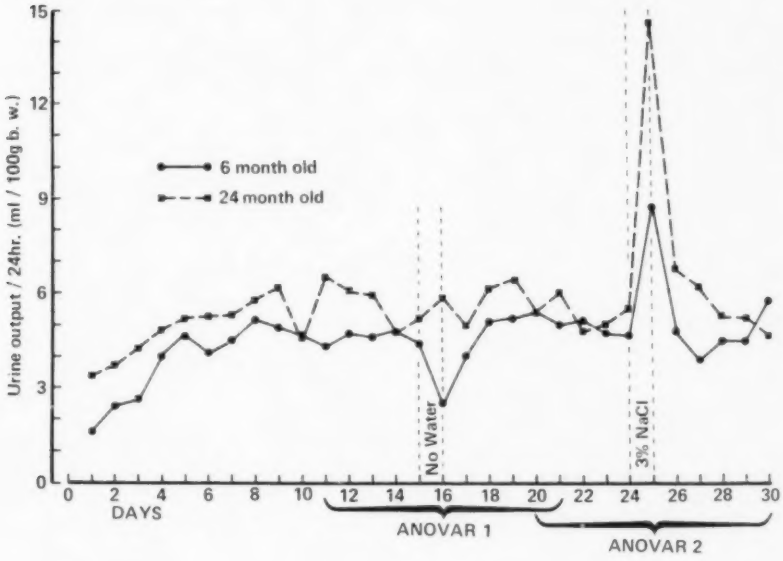

FIG. 3. *Urine output/24 hours (g/100g body weight).* Data for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	1.746, $p = 0.213$	F(1,10)	0.842, $p = 0.379$
Time effect:	F(10,110)	1.936, $p = 0.048$	F(10,110)	11.084, $p < 0.001$
Age/time interact.:	F(10,110)	1.587, $p = 0.119$	F(10,110)	2.379, $p = 0.014$

Urine analysis

Potassium (Figure 4): There was a significant age/time interaction in the total urinary potassium output (/100g body-weight/24 hours) during both the dehydration ($p = 0.029$) and osmotic loading ($p < 0.014$) stages of the study.

Sodium (Figure 5): There was a significant age/time interaction in the total output of urinary sodium (/100g body-weight/24 hours) during the dehydration phase ($p = 0.036$) but not during the osmotic loading phase ($p = 0.964$). During this latter stage, however, a highly significant time effect ($p < 0.001$) was observed.

Osmotic pressure (Figure 6): There was a significant time change in the osmotic pressure of the urine during the dehydration phase of the study ($p < 0.001$) and this was equal in both animal groups ($p = 0.494$). No significant changes were observed in either group during the osmotic loading period of the study ($p = 0.610$).

Urea (Figures 7 and 8): The age/time interaction was significant for the dehydration phase of the experiment ($p = 0.044$), indicating that the pattern of mean urea output (mmol/100g body-weight/24 hours) was not equal in the two age-groups. In the second phase, the interaction was not significant ($p = 0.217$) but there was a significant time-effect ($p < 0.001$) showing that urea-output changed with time but by the same amount in the two animal groups.

The contribution of urea to the urine osmotic pressure was also investigated by analysing the difference between the overall osmotic pressure value and the concentration of

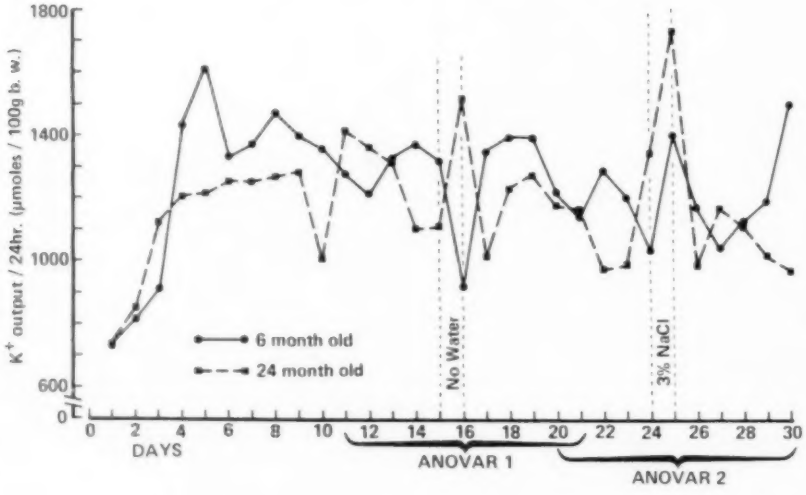

FIG. 4. *Urine Potassium output/24h. (micromoles/100g body weight)* Data for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	0.037, p = 0.851	F(1,10)	0.379, p = 0.551
Time effect:	F(10,110)	0.577, p = 0.830	F(10,110)	5.153, p < 0.001
Age/time interact.:	F(10,110)	2.114, p = 0.029	F(10,110)	4.552, p < 0.014

urea. In the dehydration phase, even though on the day of dehydration the trend in the mean osmotic pressure of the urine of the young animals was towards a decrease and that of the old towards an increase, the changes in the two groups were not significantly different ($p = 0.576$) and the overall changes with time were also non-significant ($p = 0.665$). During the osmotic challenge a significant time effect was observed ($p = 0.006$) reflecting an increased osmotic pressure on the day of the stress. The effect was the same for both groups ($p = 0.429$).

AVP (Figure 9): There was a significant time-effect over both the dehydration and osmotic loading stages of the experiment ($p < 0.001$ in both cases) of AVP output in urine (ng/100g body-weight/24 hours) and the changes were equal for both age groups ($p = 0.630$ and $p = 0.992$).

Other Chemical Investigations

These studies were performed as "spot-checks" during the experiments and they are reported because they give some indication of the urine chemistry of the animals studied (other than those variables reported above). The urine pH varied between 8 and 8.5. Protein was detected in the urine of most of the animals under study. Glucose was consistently

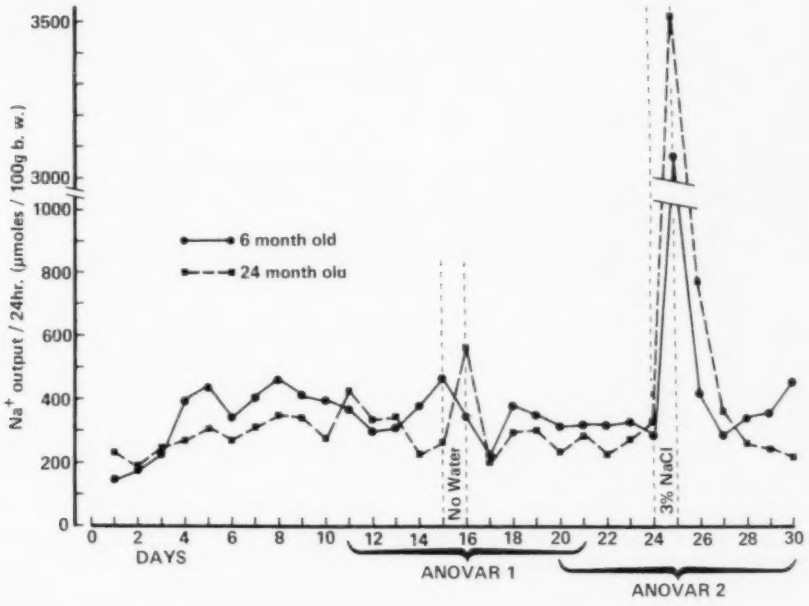

FIG. 5. *Urine Sodium output/24h. (micromoles/100g body weight).* Data for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	0.149, p = 0.707	F(1,10)	0.028, p = 0.870
Time effect:	F(10,110)	2.489, p = 0.010	F(10,110)	26.802, p < 0.001
Age/time interact.:	F(10,110)	2.038, p = 0.036	F(10,110)	0.353, p = 0.964

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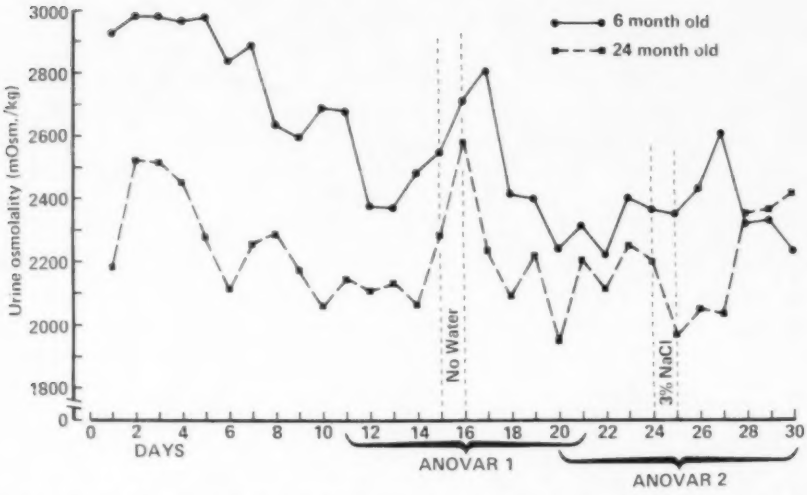

FIG. 6. *Urine osmolalities (mOsm./kg).* Data for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	1.678, $p = 0.221$	F(1,10)	0.627, $p = 0.445$
Time effect:	F(10,110)	3.868, $p < 0.001$	F(10,110)	0.820, $p = 0.610$
Age/time interact.:	F(10,110)	0.947, $p = 0.494$	F(10,110)	1.352, $p = 0.212$

negative as was ketone and blood. Microscopic examination showed a presence of occassional starch grains (possibly food contamination of the urine), and various types of phosphate crystals.

DISCUSSION

The data presented are a comprehensive view of the effects of dehydration and osmotic loading on young and old mice. A long acclimatization period is necessary before making measurements on these animals. The urinary AVP and osmotic pressure measurements were the last variables to stabilize and we feel that unless these details are recorded when studying the physiological effectiveness of the hypothalamo-neurohypophyseal-renal system then the data must be considered with reservations. Not all studies contain this information (Turkington and Everitt, 1976; Beck and Yu, 1982; Fliers and Swaab, 1983) and must therefore be treated with caution. Other investigators however, have considered the necessity for acclimatization (Bengele *et al.* 1981; Sladek *et al.* 1981), and we will discuss our results mainly in comparison with these studies.

There was a pronounced weight difference between the two age groups, with the aged animals being approximately 25% heavier. During the dehydration and osmotic loading phases of this study there was a significant age-time interaction in this variable. The data suggest a greater decrease in body weight during both the dehydration and osmotic challenge periods for the older mice. Most other studies record a similar age-related increase in body-weight (Bengele *et al.* 1981; Sladek *et al.* 1981; Beck and Yu, 1982; Fliers

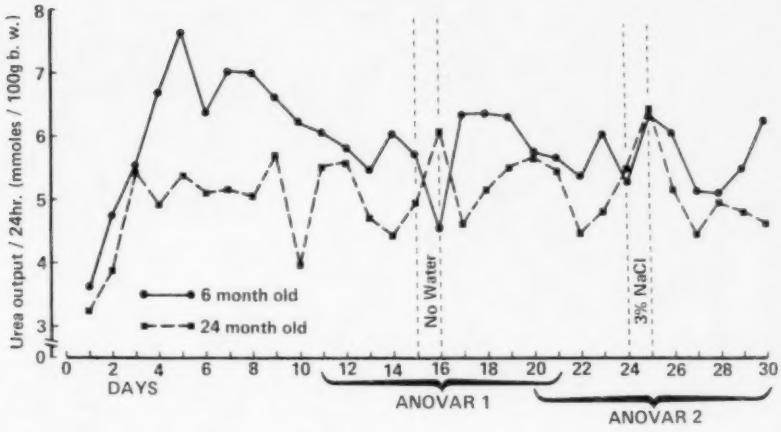

FIG. 7. *Urine urea output/24h. (mmoles/100g body weight).* Data for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	0.647, p = 0.440	F(1,10)	0.469, p = 0.509
Time effect:	F(10,110)	0.722, p = 0.702	F(10,110)	3.174, p = 0.001
Age/time interact.:	F(10,110)	1.967, p = 0.044	F(10,110)	1.342, p = 0.217

Additional Notes:

ANOVA 1: The Greenhouse-Geisser and Huynh-Feldt Frobabilities were calculated for the time-effect and the age/time interaction. The Greenhouse-Geisser probability for the time-effect was 0.6042 and the Huynh-Feldt probability was 0.7001. In the case of the age/time interaction the Greenhouse-Geisser probability was 0.1019 and the Huynh-Feldt probability was 0.0449. It was concluded that there was no significant time-effect but there was a significant age/time interaction.

ANOVA 2: The Greenhouse-Geisser and Huynh-Feldt Probabilities were calculated for the time-effect and the age/time interaction. The Greenhouse-Geisser probability for the time-effect was 0.0247 and the Huynh-Feldt probability was 0.0061. In the case of the age/time interaction the Greenhouse-Geisser probability was 0.2714 and the Huynh-Feldt probability was 0.2452. It was concluded that there was a significant time-effect but but there was no significant age/time interaction.

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and Swaab, 1983); and Sladek *et al.* (1981) observed a greater decrease in body-weight of old, dehydrated Fischer 344 rats.

There was no significant difference between the age-groups in food-intake, but a significant time-effect was recorded during the experiment. The lack of an age-time interaction implies that both age-groups control food-intake in a similar manner. It is difficult to compare the results for food-intake in our animals with those from other studies. Fliers and Swaab (1983) did not provide food for technical reasons, and Bengele *et al.* (1981) withheld food and water during the dehydration period. Other studies did not give any details of changes in this variable.

Water-intake is controlled similarly in both age-groups, implying no age-change in the solute load over this period of the lifespan in this strain of mouse. Baseline water-intake was not apparently established in either Sladek *et al.*'s (1981) or Fliers and Swaab's (1983) investigations. Bengele *et al.* (1981) showed that water-intake in terms of volume/24 hours increased with age in the Fischer 344 rat, but they did not express their observations in

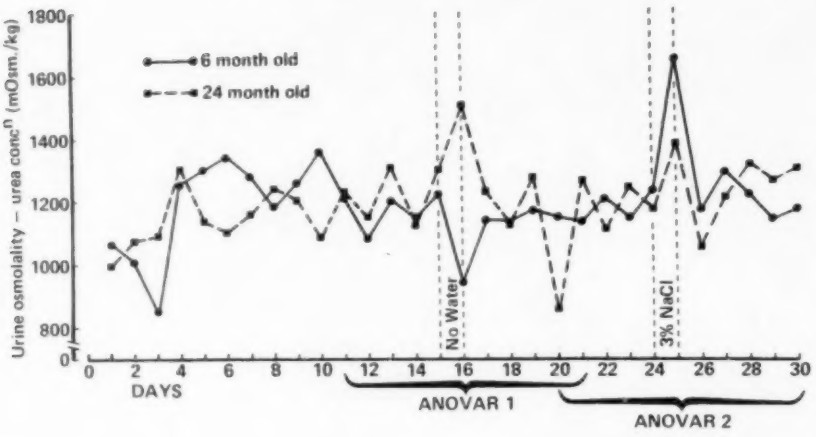

FIG. 8. Difference between total urine osmotic pressure and contribution from urea (mmoles/100g body weight) for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	0.334, p = 0.576	F(1,10)	0.001, p = 0.979
Time effect:	F(10,110)	0.762, p = 0.665	F(10,110)	2.695, p = 0.006
Age/time interact.:	F(10,110)	1.624, p = 0.109	F(10,110)	1.022, p = 0.429

Additional Notes:

ANOVA 1: The Greenhouse-Geisser and Huynh-Feldt Probabilities were calculated for the time-effect and the age/time interaction. The Greenhouse-Geisser probability for the time-effect was 0.5859 and the Huynh-Feldt probability was 0.6645. In the case of the age/time interaction the Greenhouse-Geisser probability was 0.1660 and the Huynh-Feldt probability was 0.1092. It was concluded that there was neither a significant time-effect nor an age/time interaction.

ANOVA 2: The Greenhouse-Geisser and Huynh-Feldt Probabilities were calculated for the time-effect and the age/time interaction. The Greenhouse-Geisser probability for the time-effect was 0.0341 and the Huynh-Feldt probability was 0.0067. In the case of the age/time interaction the Greenhouse-Geisser probability was 0.4115 and the Huynh-Feldt probability was 0.4286. It was concluded that there was a significant time-effect but no age/time interaction.

terms of a standardized body-weight. On the other hand Beck and Yu (1982) showed a significant increase in water-intake (corrected for body-weight) in 24-month old Fischer 344 rats when compared with 6- and 12-month-old animals. Turkington and Everitt (1976) recorded an increase in water turn-over with age (i.e., the ratio of urine output: water intake) in the male Wistar rat, but unfortunately they do not state whether or not the increase was significant, nor whether the urine-outputs and water-intakes were corrected for body-weight changes.

Faeces-output differs in the two phases of the experiment. A significant age-time interaction during the dehydration stage of the study suggests different control of this variable between young and old mice. However, there is considerable variation in the faeces-output of the old animals during this period, suggesting that it is not a stable variable. One explanation for the changes seen would be a loss of water via the faeces in the older animals, perhaps associated with some age-related alteration in the absorption of water across the gut. During the osmotic-loading stage of the experiment there is no

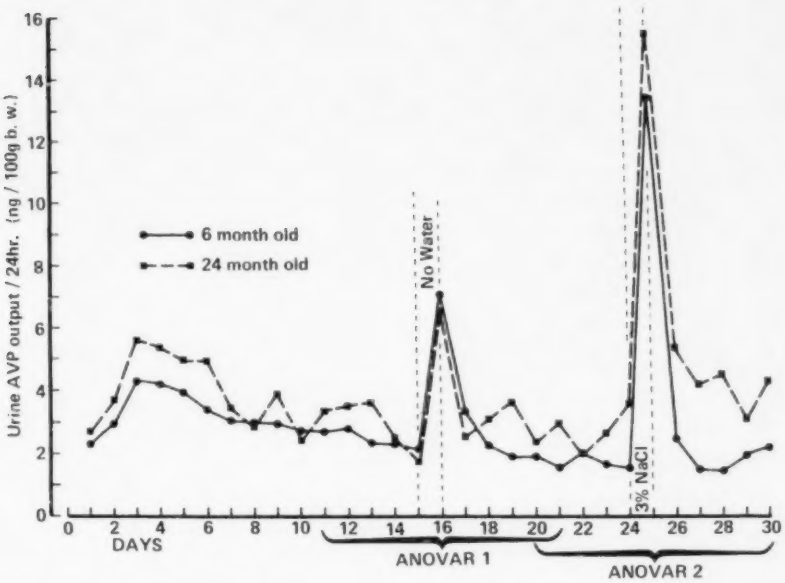

FIG. 9. *Urine AVP outputs/24h. (ng/100g body weight).* Data for the complete experiment are presented graphically with details of the ANOVA.

	ANOVA 1: Days 11 to 21, dehydration		ANOVA 2: Days 20 to 30 osmotic load	
Age effect:	F(1,10)	0.349, p = 0.566	F(1,10)	0.938, p = 0.356
Time effect:	F(10,110)	8.321, p < 0.001	F(10,110)	11.899, p < 0.001
Age/time interact.:	F(10,110)	0.798, p = 0.630	F(10,110)	0.236, p = 0.992

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significant age-time interaction in the faeces-output suggesting that the young and old mice control this variable in a similar manner during this stage of the experiment. The other studies referred to above have not recorded changes in this variable.

An analysis of urine-output during the dehydration phase of the study shows only a time-related difference during the study. Although there is opposing behavior in this variable between the young and old groups on the day of dehydration, the variance in the old age group is very great and no significant differences can be detected. In the rat, urine-output was significantly higher in old- when compared with young animals (Bengele *et al.* 1981), but in this study there was no correction for the increased body-weight of the older Fischer 344 rats. Sladek *et al.* (1981) and Beck and Yu (1982), record an age-related increase in urine-output when corrected for body-weight in rats from the same strain. Conversely, Fliers and Swaab (1983) show a significant decrease in urine volume in Wistar rats with age but they also do not correct for the increased body-weight of the older animal. Turkington and Everitt (1976) however, also used Wistar-derived animals and they observed an increase in urine-output with age (again uncorrected for body-weight changes).

The measurement of urine-output during dehydration was performed more accurately in the sophisticated studies of Beck and Yu (1982) and Bengele *et al.* (1981). Bengele *et al.* (1981) showed that during 40 hours of dehydration the solute-free water reabsorption and

osmolar clearance (expressed as a percentage of the glomerular filtration rate, GFR) were lower in older rats. However, the relationship between solute-free water formation and urine volume (both expressed as a percentage of the GFR) were similar. Since Bengele *et al.* (1981) applied supra-maximal doses of pitressin during the dehydration period it seems likely that the defects in solute-free water reabsorption, osmolar clearance and urine concentrating ability are renal in origin. One major criticism of this study is the use of pitressin to stimulate the kidney. The use of pure AVP peptide would have been more rigorous. Beck and Yu (1982) record similar findings. Turkington and Everitt (1976), however, claim that in the older, Wistar-derived rat the kidneys are as sensitive to AVP stimulation as those from young animals.

There is a significant age-time interaction for urine-volume during the osmotic loading stage of the study. It appears that during this period of hyperosmotic stimulation there is a greater osmotic diuresis and hence increased urine volume in the older groups.

Urine Analysis

The 24-hour output of potassium in the urine of these mice is extremely variable. However, a significant age-time interaction was detected in both phases of the experiment. There were opposing values for potassium excretion on the day of dehydration, and much greater output during the osmotic loading stage. The effect of age on sodium-output during 24 hours differs between the different stages of the experiment. Firstly, during dehydration there is a significant age-time interaction, suggesting that the differences here relate to the timing of the sodium-output on the day of dehydration. Thus, the old animals excrete sodium rapidly while the young mice appear not to respond. However, there is a general "crossing over" of this variable between the two age groups on days 10 to 16 suggesting that an even longer period of acclimatization may be desirable before measurement. During dehydration Beck and Yu (1982) showed a decrease in sodium concentration in the urine of old rats after 40 hours of water deprivation (and no AVP supplementation).

In the osmotic-loading phase of the study, the only effect recorded is the massive output of sodium during the administration of 3% sodium chloride for 24 hours.

Urine osmotic pressure showed a significant time-effect only after the periods of dehydration and osmotic-loading. The data shows a trend towards a lower urine osmotic pressure for the older age group but they are not significantly different from the young group. Thus, we have a paradox in that there is no significant difference between the osmotic pressure of the urines from these animals but there is a significant increase in the amount of urine volume voided during the hyperosmotic stimulation phase of the experiment. Basal urine osmotic pressure was shown to decrease with age in the Fischer 344 rat (Bengele *et al.* 1981; Sladek *et al.* 1981; Beck and Yu, 1982). In addition, under conditions of water deprivation it was shown that the urine concentrating ability was impaired in the old Fischer 344 animal (Beck and Yu, 1982; Benegle *et al.* 1981), although in an experiment comparable with the one conducted here Sladek *et al.* (1981) showed that the old Fischer 344 rat was capable of mounting a significant anti-diuresis.

The measurement of the output of urea in the urine showed a significant age-effect in only the dehydration phase of the experiment. In the young mice there was trend towards a decreased urine-output during dehydration, and in addition, there was a decrease in the output of urea over the 24 hour period. The old animals on the other hand do not appear to decrease the output of urine, but they do increase the excretion of urea, thus explaining

the paradox raised by the increased osmotic pressure found in an unchanged urine volume. It has been suggested that the control of the excretion of urea is mediated by the action of AVP. A simple explanation of our findings is that in the young animal AVP is acting on a responsive kidney, which therefore lowers the output of urine. Simultaneously there is a reduced output of urea. In the older mouse there is a decreased sensitivity of the kidney to the action of AVP (Goddard *et al.* 1983; Goddard *et al.*, in press), urine volume is not reduced, and urea output continues unabated. Since there is also a net loss of body-weight during the dehydration period of the experiment (mainly due to a loss of body-water), the old animals must also be undergoing an increase in the concentration of urea in the blood, an additional form of control over this process would be the active loss of urea through the kidney. It is tempting to speculate that over a prolonged period of dehydration there could be an irreparable alteration of the medullary solute gradient in the aged kidney because of the severe loss of essential components.

During the osmotic challenge period of the study there is no age-effect but there is a marked time-effect observed in urea output, with a pronounced decrease apparent in both age-groups on the day of the osmotic load.

The effect of urea on the urine osmotic pressure was also investigated. The urea component was subtracted from the overall osmotic pressure and in the dehydration stage of the study there was no significant alteration in osmotic pressure recorded with either age or time, suggesting that the major component of the osmotic pressure change in the uncorrected data was due to urea. The simple interpretation that we have adopted is that the alteration in urea output is a reflection of the effectiveness of AVP on water conservation in the kidney. While we have no statistically significant data, the trends are towards a reduction in the volume of urine in the young mouse with a concommitant decrease in urea output. In the older mouse there is no apparent reduction of urine volume but there is an increase in the osmotic pressure of the urine.

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During an osmotic challenge there is a net osmotic diuresis and under these conditions both young and old mice increase the output of urea in the urine.

Of particular interest to us was the measurement of AVP in the urine during the experiment. After the initial high output in the older animals, possibly associated with the stress of being put into metabolic cages, the levels stabilize. No age-differences could be detected. Similarly, during both the dehydration and osmotic challenge there were no age-related differences detectable, yet both groups excreted very large and similar quantities of AVP. Turkington and Everitt (1976) showed a decrease in the output of AVP in the urine of old Wistar rats. However, without a correction for body-weight changes with age in this strain the results are meaningless. A recent paper (Matsui *et al.* 1983) shows a very high correlation between the concentration of AVP in blood samples collected over a period of 30 minutes and concurrent urinary excretion of AVP, indicating the utility of using changes in the excreted AVP as an index in change in plasma AVP concentration. These authors go on to suggest that the concentration of AVP in a single blood sample, taken over a brief period of time, may provide a very poor index of AVP secretion in view of the reported episodic release of AVP from the neurohypophysis (Weitzman *et al.* 1977). Thus, the urinary excretion of AVP may provide a more reliable integrated index of AVP release provided there are no substantial changes in renal haemodynamics (Matsui *et al.* 1983). However, even if the GFR falls, so reducing the glomerular clearance of AVP, peritubular clearance increases and the total clearance of AVP by the kidney remains constant (Rabkin *et al.* 1979).

It can be concluded that during the period of the lifespan studied there is no age-related

alteration in the output of AVP from the neurohypophysis, either during normal hydration, dehydration or hyperosmotic challenge. The intake of water does not alter during the period investigated nor do we detect significant alterations in the output of urine in older mice. However, it is clear that there are age-related differences in the control of sodium, potassium and urea outputs in the urine. The trends in these data are towards an age-related decline in kidney function, but the variance in the older groups may be masking significant alterations. Further investigations of all these variables are continuing in older animals.

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EFFECT OF AGE AND MYOCARDIAL INFARCTION ON SERUM AND HEART LACTIC DEHYDROGENASE

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Abstract—The effect of isoproterenol (ISO) on the activity of lactic dehydrogenase (LDH) in the serum and heart was investigated in rats belonging to four different age groups. Serum LDH activity increased in all the ages as a result of ISO injection, however, the magnitude and response varied with age. Younger animals were able to recover from the toxicity of ISO much quicker compared to the older ones. An elevation in the LDH enzyme activity in the serum correlated with a decrease in the activity of cardiac muscle LDH in all the age groups of isoproterenol-treated rats.

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INTRODUCTION

LACTIC DEHYDROGENASE (LDH) level remains abnormal for nearly 120 hours after an infarction. Hence serum LDH determinations are very useful in diagnosing myocardial infarction when the exact time of onset is not known and in cases where hospital admissions are delayed (Bauman, 1980; Neufeld *et al.* 1977). An increase in serum malic and lactic dehydrogenase following an acute infarction of the heart has been reported (Wacker *et al.* 1956). Effects of isoproterenol (ISO) and the activity of LDH in serum and cardiac muscle as a function of age is not known and is the subject of this paper.

MATERIALS AND METHODS

Rats (Wistar strain) of 20, 40, 60 and 80 weeks of age were injected with isoproterenol hydrochloride (ISO) in saline 30 mg/100 g body weight and controls with 0.9% saline. The rats were sacrificed at 4, 8, 12, 24 and 48 hours following the administration of ISO.

The normal and infarcted hearts were isolated, weighed and washed in 0.9% saline. A 5% (w/v) homogenate was prepared in ice-cold 0.1M phosphate buffer (pH 7.2) and centrifuged at 6000 rpm for 20 minutes at 4 °C. The extraction was repeated twice and the supernatants were pooled for the assay. The residue did not contain any detectable amount of LDH activity and hence was discarded. Blood was drawn from the heart, allowed to clot on ice and centrifuged at 0 °C for 10 minutes at 3000 rpm to separate the serum. Hemolyzed samples were discarded. Lactic dehydrogenase activity was estimated as per Alan (1961) with lithium lactate as the substrate and the activity was expressed as units/ml serum or muscle homogenate.

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TABLE 1. CHANGES IN SERUM LDH ACTIVITY DUE TO ISOPROTERENOL INJECTION IN DIFFERENT AGE GROUPS OF RATS.

Age (weeks)	*Control*	*Hours after injection of isoproterenol*				
		4	*8*	*12*	*24*	*48*
20	65.43 ± 10.40	125.20 ± 16.70*	148.32 ± 20.05*	182.80 ± 14.80*	136.00 ± 12.56*	124.00 ± 10.20*
40	90.05 ± 10.00	120.40 ± 6.50@	142.80 ± 11.08@	179.40 ± 9.02*	181.60 ± 18.00*	190.00 ± 20.02*
60	152.67 ± 6.19	197.67 ± 18.01*	213.89 ± 11.06*	244.98 ± 15.67*	277.67 ± 23.90*	278.00 ± 40.01@
80	160.80 ± 12.95	192.96 ± 12.66!	219.04 ± 20.10@	241.20 ± 40.20@	260.49 ± 20.80*	292.75 ± 12.36*

Activity of LDH is expressed as units/ml serum and values are means ± standard deviation of four observations.

*p < 0.001 @p < 0.01 !p < 0.05

RESULTS

Significant decrease in the activity of cardiac LDH and increase in serum LDH were seen 4 hours after the administration of isoproterenol, effects being very significant until 60 weeks of age. Serum LDH increased due to ISO injection in all the four groups. Max-

TABLE 2. CHANGES IN CARDIAC LDH ACTIVITY AS A FUNCTION OF ISOPROTERENOL INJECTION IN DIFFERENT AGE GROUPS OF RATS.

Age (weeks)	*Control*	*Hours after injection of isoproterenol*				
		4	*8*	*12*	*24*	*48*
20	470.31 ± 24.50	255.18 ± 22.20*	242.04 ± 24.60*	217.29 ± 25.86*	381.75 ± 57.00!	430.50 ± 15.00'
40	838.50 ± 16.99	436.02 ± 35.02*	383.94 ± 30.60*	335.40 ± 21.50*	209.70 ± 12.50*	201.30 ± 11.80*
60	1152.00 ± 75.50	533.28 ± 44.00*	507.96 ± 23.65*	407.02 ± 22.85*	288.00 ± 31.46*	285.00 ± 21.40*
80	814.20 ± 12.90	367.21 ± 14.60*	353.77 ± 42.00*	284.97 ± 18.90*	280.00 ± 10.50*	380.60 ± 15.00*

Activities of LDH are expressed as units/ml homogenate and are means ± standard deviation of four observations.

*p < 0.001 !p < 0.05 'Not significant

imum (180%) increase was seen at 12 hours after ISO injection and a decline (89%) by 48 hours was seen in the 20-week-old. In the 40-week-old rat the activity increased from 90 to 179 units within 12 hours (100%), and in the 60-week-old rat the increase was seen until 24 hours, whereas in the 80-week-old rat the increase persisted until 48 hours (Table 1).

Due to the administration of isoproterenol, LDH activity decreased in cardiac muscle in all the four age groups studied (Table 2). In the 20-week-old rats the enzyme activity decreased up to 12 hours, whereas in 40-, 60- and 80-week-old rats the activity decreased up to 24 hours. It is significant that the LDH activity increased after 12 hours in the 20-week old, almost reaching the control values by 48 hours. Corresponding to the serum LDH increase as a function of isoproterenol injection, a sharp decrease in the cardiac LDH and in the ratio of tissue/serum activity were prominent. In all the age groups, the decrease in the ratio of tissue/serum LDH was noticeable within 4 hours after injection of ISO. However, the ratio decreased up to 12 hours with the 20-week-olds, whereas in the older groups the ratio decreased up to 48 hours.

DISCUSSION

Lactic dehydrogenase is used as one of the important diagnostic markers in the evaluation and confirmation of myocardial infarction. LDH activity in the cardiac muscle showed a decline during the first 12 hours after isoproterenol injection and thereafter an increase was seen (until 48 hours) in the 20-week-old rat. This is in agreement with the results of Ganguly *et al.* (1980). Animal experiments have shown that the ischaemic region of the heart suffers a depletion of several enzymes and these in turn appear in the serum (Agress *et al.* 1955; Jennings *et al.* 1975; Lemley-Stone *et al.* 1955; Nydick *et al.* 1955). A correlation between the rise in the activity of individual enzymes in serum and the appearance of tissue damage in the heart muscle have been indicated (Sadamachi, 1981; Visser *et al.* 1981). A 21% increase in the enzyme activity in dogs subjected to experimental infarction by ligation of several branches of the coronary artery has been reported (Gudbjaranason and Priver, 1968). The possibility of loss in LDH by efflux from the myocardium has been suggested (Johnson *et al.* 1981) in their studies with coronary artery ligation in dogs. In the present results, the percent decrease in cardiac LDH activity, however, was greater in the older rats, and they exhibited lesser elevations in serum LDH activity compared to the young. Our earlier studies have shown a similar inverse relationship in the case of alanine aminotransferase as a function of exercise and age (Asha and Radha, 1981).

Rise in the serum enzymes after an infarction is due to the release of these enzymes from the infarcted cells into the blood stream and is proportional to the extent of tissue damage (Wilkinson, 1970). Serum level is elevated for a much longer time than any other enzyme and younger rats show a better response to isoproterenol compared to older rats (Wexler, 1978).

Our investigations clearly show that there are definite age-specific responses to isoproterenol and the younger animals have a better ability to recover quickly and almost completely from the cardiotoxic effects of isoproterenol compared to the older group.

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SPECIAL ARTICLE

GERONTISM: A NEOLOGISM

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GERIATRICS AND *gerontology* as the major headings for the science of *aging* do not preclude, and may even invite, the introduction of new terms to designate those characteristics of *senescence* that need new labels.

Words about *aging*, *old age*, and the *aged* are of two dominant etymological origins. Those from the Greek have been preempted in large measure by the scientific disciplines, while those of Latin derivation are more familiar in the public domain.

The Greek root *ger* is the basis for *geras*, old age, *geron*, old man and plural, *gerontes*. Geriatrics (1909) (*geras* and *iatrikos*, medical healing of) refers to the clinical aspects of aging (Nascher, 1909, 1911, 1940). Gerontology (1903) (*gerontes* with *logos*, discourse or science of) refers to the biological features of *senescent processes* (Metchnikoff, 1903, translation by Chalmers, 1908). The galenic term *gerokomy* (*gerocomy*, *gerokomia*, *gerentocomia*), (1st century and later) (Smith, 1666; Floyer, 1724; Zerbi, 1489) is a combination of *geras* with *komein*, tending or nursing care of, in which individual efforts for an old man have been extrapolated to mean the sociology of aging in the sense of society in its entirety acting as supportive agency for all of its elderly (Freeman, 1954). Such sociologists would be called *gerocomists*. Other constructions such as *geraeology* or *gereology* did not take hold as the result of the failure to get support for this very proper grammatical construction of *geras* and *logos*. *Geratology* suffered the same fate when it was displaced by the common acceptance of gerontology. *Gerontophilia*, *gerontocracy*, *gerontophobia* and a growing number of combined words such as the unnecessary *clinical gerontology* as a substitute for *geriatrics* have proliferated in an effort to identify subspecies of thought about aging.

The Latin root *sen* for *senex* and *senium* gave rise to *senescence* (process of aging), *senility* (state of old age), *senectitude* (abject status of age), *senate*, *senator*, *senilism* and even *senior citizen*, which is a popular form that is not likely to be used by professionals in the field.

Ageism (Butler, 1969) originated from the Latin *aetas* (age), which became *aäge* in the earlier French and *âge* in its later form, finally entering English as *age*. The fairly recent

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combination of *age* and *ism* has practically displaced *senilism*, *agedness* and *senectitude* as a label for the prejudices against the old. The implication is that the old as a group consist of a hapless cohort that needs defenses against socioeconomic and even personal forces that take advantage of, or hold a bias against, the older fraction of society. *Ageism* carries a sense of derogation, probably from its birth in anger at perceived inequities, but leavened by the statement that "anger," according to Shakespeare "hath a privilege." These privileges have a tendency to become exceeded. The very real need for the term *ageism* requires an even greater need for a calmer, considerate, and equable way of referring to *old age* in a way that is free of hostility, deprecation, frustration, and blanket charges of neglect.

The combination of *geron* and *ism* as *gerontism* is an appropriate title for the comprehensive concepts of all aspects of *aging*. (*Ageism* is not an antonym for *gerontism* just as *geriatrics* is not a synonym for *senility*). The new word is a response to the need for the way in which all features of *aging* can be considered without a hint of prejudice or the broad call to arms that seems to be implicit in *ageism*. By its acceptance, the overtones of the latter, useful as it is in the identification of deficiences in attitudes, are dispelled by a new generic term free of J'Accuseism while affording a rather useful noun for responsible students of *aging* who need *ageism* as part of their vocabulary.

The grammatical forms of *gerontism* follow readily. Students of *gerontism* are *gerontists*. The adverb is *gerontally*. Adjectives already found in dictionaries are *gerontic* and *gerontal* which should displace the incorrect use of *geriatrics* as an adjective in any form other than as a clinical reference.

If the term *gerontism* is accepted, major words for the headings of *aging*, excepting some combined forms, would be: *geriatrics*, for the clinician; *gerontology*, for the biologist; *gerocomy*, for the sociologist; *ageism*, for its own needs; and for everyone else, *gerontism* as the heading under which all of the issues of *aging* could be marshalled without prejudice, implied or implicit.

Addendum—The term *gerontism* was used in a publication for the first time July 15, 1983 (Freeman, 1983). It was repeated in the presentation *A Geriatric Odyssey* by Freeman, given at the annual meeting of the Gerontological Society of America in San Antonio, Texas, November 18, 1984 as the annual Clinical Lectureship in Geriatrics (Freeman, 1984) on nomination by the Society's Clinical Medicine Section.

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AGING OF MEMBRANE TRANSPORT MECHANISMS IN THE CENTRAL NERVOUS SYSTEM—HIGH AFFINITY CHOLINE TRANSPORT IN RAT CORTICAL SYNAPTOSOMES

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Abstract—High affinity choline transport has been studied in cortical synaptosomes from rats ranging from 2 months to 30 months of age. Initial velocities were measured as a function of both choline and sodium concentration. These data give similar fits to a kinetic model which was previously found to give minimal best fit to data from 2-month-old animals. Thus, aging has apparently not altered the fundamental mechanism by which carrier, sodium, and choline interact in the process of uptake. Although some small differences in initial velocities were found between data from young and older animals, these differences showed no pattern and were not statistically significant. It is concluded that the choline transporter is unaffected by age, in contrast to the transporters for GABA and glutamic acid, whose transport capacities were found to decline with age.

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INTRODUCTION

A PREDOMINANT feature of aging is the loss of homeostatic regulation (von Hahn, 1970). Because the nervous system plays such a vital role in homeostasis, decline in CNS function with age is likely to be integral to the loss of regulatory ability (Franks, 1970; Sohal and Sharma, 1972; Hayflick, 1976; Diamond, 1978; Lytle and Altar, 1979). Evidence is accumulating that changes in synaptic transmission are contributory to this decline in nervous system regulatory capacity (Kent, 1976; Pradhan, 1980). Membrane transport mechanisms are essential to the synaptic role of many neurotransmitters.

In previous studies of the uptake of the putative transmitters glutamic acid and GABA in rat cortical synaptosomes (Wheeler, 1980; 1982) and GABA in hypothalamic synaptosomes (Wheeler, 1983), initial velocities and maximal transport capacities were found to decline with age. Although the same model was found to fit data from both young and aged animals, changes were found in the kinetic constants which quantitate the model. Alterations with age are not common to all transport mechanisms, however; similar studies of the glucose transporter showed no change with age (Wheeler, 1981). Uptake of glucose is via a low affinity, sodium independent mechanism, which is not directly related to synaptic function (Wheeler and Hollingsworth, 1979a), while uptakes of glutamate (Wheeler

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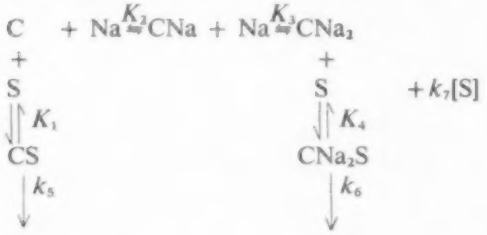
and Hollingsworth, 1978) and GABA (Wheeler and Hollingsworth, 1979b) are via high affinity, sodium dependent mechanisms which are thought to be integral to the synaptic function of these compounds.

Choline, necessary for acetylcholine synthesis (Birks and MacIntosh, 1961; Collier and MacIntosh, 1969), cannot be synthesized by nervous tissue (Birks and MacIntosh, 1961). Thus, the nerve cell must accumulate choline from the extracellular fluid. This is accomplished by a high affinity, sodium dependent transport mechanism (Kuhar and Murrin, 1978).

The kinetics of the interaction of sodium and choline with the carrier have been studied in this laboratory (Wheeler, 1979). Initial velocities were measured as a function of both choline and sodium concentrations, and these data were then fitted to the rate equation for each of several models which might account for the interaction of sodium and choline with the transporter. The following model gave minimal best fit to the data:



(1)

C = carrier
S = choline
Na = sodium
$K_1 - K_4$ = dissociation constants
k_5, k_6 = translocation constants
k_7 = permeability constant

At normal [Na], most of the uptake takes place by the carrier mechanism (the exact fraction is dependent on the substrate concentration), and about 99% of that uptake is via the CNa_2S leg of the model.

As pointed out above, membrane transport mechanisms, including that of choline, are essential to the synaptic role of many transmitters. Which of these mechanisms change with age? Do all transport mechanisms which play a role in synaptic transmission change with age? Do all high affinity or sodium dependent mechanisms change with age? The present studies of choline transport were undertaken to help answer these questions.

MATERIALS AND METHODS

Male Long-Evans rats 2 to 3, 10 to 11, 18 to 19 or 29 to 30 months-old were used in this study. These rats were housed two per cage under conditions of constant temperature and controlled illumination, with *ad libitum* access to food and water. The diet was obtained from Wayne Feeds (Allied Mills, Inc., Libertyville, IL).

An extensive study of the suitability for membrane transport studies of the preparation and techniques used in the present study has been published (Wheeler, 1978). The methods used in the present research evolved from this study. Because the details of the methods used have been published (Wheeler, 1979), only an abbreviated outline is given.

Preparation of synaptosomes

Cortical tissues were homogenized in 20 volumes of ice cold 0.32 *M* sucrose, and homogenates were centrifuged for 10 min at 1085 *g* (2°C) to remove nuclei and cellular debris. The synaptosomes were then sedimented by centrifugation at 27,000 *g* for 15 min. The supernate was discarded, and the synaptosomes were resuspended in cold sucrose.

Measurement of uptake

Uptake of 14-C labelled choline was measured by incubation of synaptosomes for four or eight minutes in a modified Krebs–Henseleit solution at 30°C (Lockwood, 1961). All incubations were carried out in a Dubnoff metabolic shaker. The concentration of choline in the final incubation medium was 5.0, 2.0, 1.0, 0.6667, or $0.5 \times 10^{-6} M$. Concentration of sodium in the incubation medium was varied by replacement of NaCl with equiosmolar Tris buffer; 0, 19.0, 27.0, 43.0, and 120.8 m*M* sodium solutions were used with each of the above choline concentrations. Following incubation, synaptosomes were recovered on Millipore filters, washed, and the filters blotted and counted for radioactivity. Single measurements of initial velocities for each of the choline concentrations, along with each of the several sodium concentrations, were made with synaptosomes from a single preparation of tissue from one animal. For 4-minute uptakes, the measurements were repeated on 11, 12, and 12 animals for the 2-, 18-, and 30-month age groups, respectively. For 8-minute uptakes, the measurements were repeated on 6 animals in each age group. Four- and eight-minute uptakes were done on separate groups of animals.

Computer optimization

After correction for filter retention of label when incubation medium was poured through them; initial velocities were calculated, and these data were fitted to the velocity equation. A parameter optimization algorithm based on the Flexible Polyhedron approach of Nelder and Mead (1965) was used to minimize the following function:

$$J = \sum_{i=1}^{n} \left(\frac{V_{\text{model}} - V_{\text{data}}}{V_{\text{data}}} \right)^2 \tag{2}$$

J, the objective function, is the sum of the squared fractional errors between the velocities of uptake predicted by the equation (V_{model}) and those observed experimentally (V_{data}).

Statistical analysis

Analysis of variance was carried out with the use of Human Systems Dynamics' ANOVA II program and the Apple II+ computer.

RESULTS

Comparison of initial velocities

Two sets of initial velocity measurements were made. In the first set of experiments, 4-minute uptakes were measured on aliquots of synaptosomes equivalent to 10 mg tissue wet weight from animals 2-, 18-, and 30-months of age (Table 1). Although there are some differences among the age groups, these differences are usually small and do not exhibit a pattern. For example, at [Na] = 120.8 m*M*, uptake is slightly less in most instances for the older animals, while at [Na] = 0, uptake is greater in most cases in the older groups. A multifactor analysis of variance was done on the data; although the sodium and choline effects are highly significant, there is no significant effect of age ($P > .05$).

Because of the scatter in the data of Table 1, a second set of velocity measurements was made. Incubation time and aliquot size were each doubled in order to increase the

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TABLE 1. EFFECT OF AGE ON 4-MINUTE UPTAKE

[Choline] × 10⁶,M		*0.5*	*0.6̄*	*1.0*	*2.0*	*5.0*
[Na], m*M*	Age					
0	2 M	0.10 ± .06	0.18 ± .09	0.25 ± .11	0.87 ± .09	1.75 ± .23
	18 M	0.16 ± .02	0.22 ± .04	0.50 ± .06	0.58 ± .07	1.64 ± .35
	30 M	0.15 ± .02	0.32 ± .04	0.50 ± .07	0.75 ± .08	1.50 ± .27
19	2 M	0.18 ± .06	0.26 ± .08	0.47 ± .07	0.98 ± .08	2.15 ± .18
	18 M	0.33 ± .04	0.40 ± .05	0.61 ± .06	0.93 ± .08	2.11 ± .22
	30 M	0.33 ± .04	0.45 ± .09	0.78 ± .10	0.96 ± .12	1.88 ± .34
27	2 M	0.30 ± .05	0.44 ± .04	0.65 ± .08	1.35 ± .06	2.70 ± .16
	18 M	0.40 ± .04	0.50 ± .04	0.79 ± .05	1.25 ± .11	3.14 ± .29
	30 M	0.36 ± .05	0.61 ± .05	0.73 ± .09	1.18 ± .11	3.31 ± .35
43	2 M	0.46 ± .06	0.66 ± .11	0.85 ± .13	1.69 ± .08	3.65 ± .36
	18 M	0.51 ± .03	0.60 ± .04	0.86 ± .03	1.56 ± .10	2.97 ± .21
	30 M	0.49 ± .04	0.67 ± .04	0.97 ± .07	1.65 ± .14	2.75 ± .27
120.8	2 M	1.73 ± .07	2.02 ± .10	2.62 ± .14	3.95 ± .19	6.16 ± .20
	18 M	1.64 ± .06	1.87 ± .08	2.23 ± .07	3.41 ± .14	5.48 ± .30
	30 M	1.86 ± .10	2.04 ± .09	2.46 ± .09	3.79 ± .18	5.57 ± .33

Four-minute uptakes were measured in aliquots of synaptosomes equivalent to 10 mg tissue wet weight. Synaptosomes were separated from incubation medium with 0.45 μ filters. Data represent mean values ± S.E.M. after having normalized to mg tissue • minute. Thus, the units are 10^{-13} moles/mg • minute.

N = 11, 12, and 12 for the 2-, 18-, and 30-month age groups, respectively. The 2-month age group includes data from the 5 animals in the original study (Wheeler, 1979).

magnitude of the uptake measurements (Table 2). Note that the scatter in the data of Table 2 is much less than that in Table 1. As with the data of Table 1, the differences among the age groups are very minimal; analysis of variance showed no significant effect of age ($P > .05$).

Best fit model

Figure 1 shows plots of 8-minute velocity measurements (normalized to mg tissue • minute) as a function of [Na] for 30-month-old animals. Although not shown, the shapes of the curves for all age groups are similar (both 4- and 8-minute uptakes) and all are similar to such plots from the previous study of 2-month animals (Wheeler, 1979). Although there is some uptake in the absence of sodium, uptake increases rapidly along an S-shaped curve as the [Na] is increased. The sigmoidal shape of the curves indicates that two or more sodium ions are co-transported with each choline via one of the translocation steps. As was found in previous studies, plots of velocity versus substrate concentration for all age groups fail to saturate at higher substrate concentrations, thus indicating that a linear, non-saturable component is involved (Figure 2). Thus the results from the older animals are consistent with those found previously for young animals.

The following model was found to give the minimal best fit to data in the previous study of young animals (Wheeler, 1979):

TABLE 2. EFFECT OF AGE ON 8-MINUTE UPTAKE

[Choline] × 10⁶,M		*0.5*	*0.6̄*	*1.0*	*2.0*	*5.0*
[Na], m*M*	Age					
0	2 M	0.19 ± .02	0.22 ± .01	0.32 ± .02	0.70 ± .02	1.62 ± .08
	10 M	0.19 ± .01	0.20 ± .01	0.34 ± .01	0.66 ± .02	1.54 ± .09
	30 M	0.20 ± .01	0.26 ± .02	0.35 ± .03	0.80 ± .06	1.66 ± .16
19	2 M	0.25 ± .00	0.31 ± .01	0.44 ± .02	0.95 ± .05	1.77 ± .09
	10 M	0.27 ± .01	0.30 ± .01	0.46 ± .02	0.90 ± .04	2.01 ± .13
	30 M	0.28 ± .01	0.33 ± .02	0.49 ± .02	0.97 ± .06	1.93 ± .14
27	2 M	0.32 ± .01	0.39 ± .02	0.58 ± .02	1.06 ± .06	2.00 ± .09
	10 M	0.33 ± .02	0.40 ± .02	0.64 ± .02	1.04 ± .04	2.03 ± .08
	30 M	0.35 ± .00	0.43 ± .01	0.63 ± .01	1.10 ± .05	2.21 ± .11
43	2 M	0.50 ± .02	0.64 ± .03	0.88 ± .04	1.50 ± .04	2.77 ± .14
	10 M	0.54 ± .02	0.67 ± .02	0.95 ± .04	1.57 ± .05	2.95 ± .15
	30 M	0.45 ± .02	0.67 ± .02	0.91 ± .02	1.60 ± .04	3.02 ± .09
120.8	2 M	1.56 ± .08	1.91 ± .09	2.39 ± .12	3.35 ± .14	4.97 ± .18
	10 M	1.54 ± .11	2.23 ± .19	2.51 ± .11	3.58 ± .17	5.33 ± .17
	30 M	1.50 ± .10	1.88 ± .06	2.58 ± .09	3.60 ± .08	5.33 ± .10

Eight-minute uptakes were measured in aliquots of synaptosomes equivalent to 20 mg tissue wet weight. Synaptosomes were separated from incubation medium with 0.65 μ filters. Data represent mean values ± S.E.M. after having normalized to mg tissue • minute.

$N = 6$ for each age group. Units of velocity are 10^{-13} moles/mg • minute.

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$$\begin{array}{ccccc} C & + Na \overset{K_2}{\rightleftharpoons} CNa & + Na \overset{K_3}{\rightleftharpoons} CNa_2 & & \\ + & & + & & \\ S & & S & & + k_7'[S] \\ \Big\downarrow\Big\uparrow K_1 & & \Big\downarrow\Big\uparrow K_4 & & \\ CS & & CNa_2S & & \\ \Big\downarrow k_5 & & \Big\downarrow k_6 & & \end{array} \qquad (3)$$

$$v = \frac{\dfrac{K_5[S]}{K_1} + \dfrac{K_6[Na]^2[S]}{K_2K_3K_4}}{1 + \dfrac{[S]}{K_1} + \dfrac{[Na]^2[S]}{K_2K_3K_4} + \dfrac{[Na]}{K_2} + \dfrac{[Na]^2}{K_2K_3}}$$

The shapes of the curves of Figures 1 and 2 indicate that these data might also fit this model. Because the scatter in the data for 8-minute uptakes is less than that for 4-minute uptakes, these data for 2-, 10-, and 30-month animals were fitted to the above model. Although slightly better for the 2-month age group, the fits of the model to the data are also good for the aged animals; the average percentage error per data point between experimental data and model predictions is 2.82, 4.24, and 3.17 for 2-, 10-, and 30-month animals, respectively. Thus, aging has not altered the basic mechanism by which carrier, sodium, and choline interact in the process of transport.

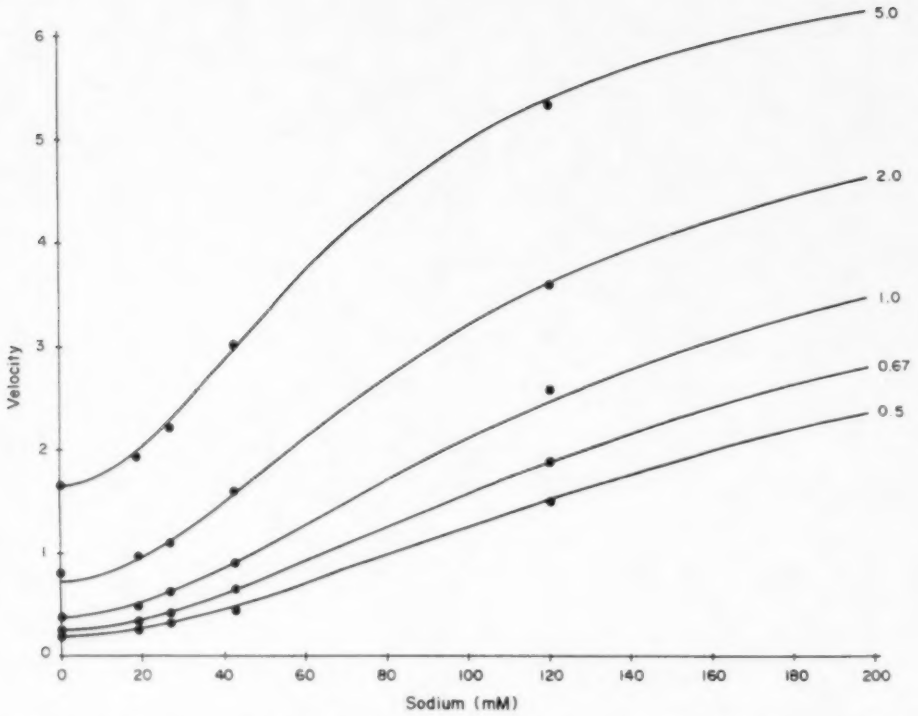

FIG. 1. Velocity as a function of sodium concentration for each choline concentration used. Symbols show experimental data points, while solid lines show computed fit to the rate equation for the best fit model. Choline concentration (μM) is given to the right of each curve. Data shown are for 30-month age group (8-minute uptakes normalized to mg tissue minute), although all data sets give similar plots.

DISCUSSION

Plots of initial velocity as a function of either sodium or choline concentration were found to be similar for all age groups, thus suggesting that the nature of the interaction of sodium and choline with carrier does not change with age. This was confirmed by the fact that the data for all age groups fit the model found previously to give minimal best fit to data from 2-month animals equally well.

Although small differences in initial velocity were found among age groups, these differences are not statistically significant. The choline concentrations used ($0.5-5$ μM) were such that uptake should have occurred primarily by the so-called high affinity carrier. Thus, high affinity choline uptake in rat cortical synaptosomes is apparently resistant to any deleterious consequences of aging. This is in contrast to similar studies of other substrates which have been carried out in this laboratory. In studies of both glutamic acid (Wheeler, 1980) and GABA (Wheeler, 1982) transport in rat cortical synaptosomes, and GABA transport in hypothalamic synaptosomes (Wheeler, 1983), significant declines in initial velocities were found in the 30-month age group. Maximal transport capabilities were also found to decline with age. However, as in the present study, no change was found in the mechanism by which carrier, sodium, and substrate interact in the process of transport.

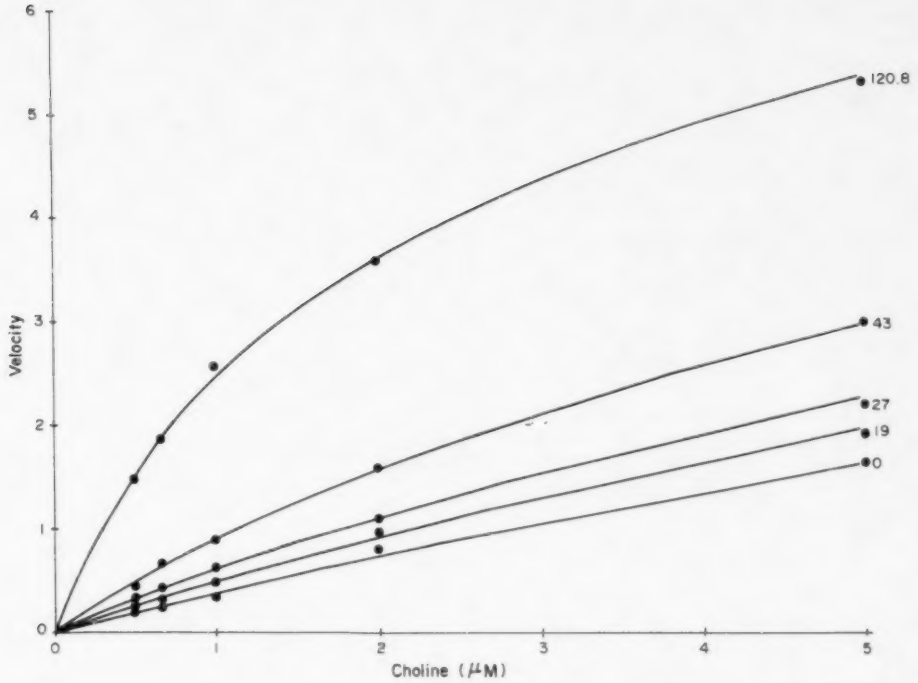

FIG. 2. Velocity as a function of choline concentration for each sodium concentration. Symbols show experimental data points, while solid lines show computed fit. Sodium concentration (m*M*) is given to the right of each curve. Data shown are for 30-month age group (8-minute uptakes normalized to mg tissue minute), although all data sets give similar plots.

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In a study of the glucose transporter, no changes were found to occur with age (Wheeler, 1981). This glucose transport mechanism, which is thought to be common to all nerve cells, is a low affinity, sodium independent mechanism which functions in maintaining cellular glucose levels essential to the metabolic role of glucose. In contrast, glutamic acid and GABA, which are thought to function as neural transmitters, are taken up by high affinity, sodium dependent mechanisms which are thought to be integral to their synaptic function. Choline uptake is also via a high affinity, sodium dependent mechanism, which is necessary in obtaining substrate for acetylcholine synthesis. Thus, aging of transport mechanisms is not common to all transporters, nor is it common to all high affinity or sodium dependent systems.

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EVIDENCE OF SCHWANN CELL DEGENERATION IN THE AGING MOUSE MOTOR END-PLATE REGION

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Abstract—An ultrastructural evaluation was made of the changes encountered in the motor end-plates of the gastrocnemius muscle of old mice in comparison with young-adult mice. In old, 27-month-old mice, a great variety of changes were recorded. The main abnormalities occurred in the axon terminals, the myoneural junctional folds, and the endomysial space. Loss of synaptic vesicles and mitochondria and vacuolization of axon terminals were common. Shrinkage and degenerative changes of axon terminals were noticed. The end-plate abnormalities were closely associated with an increased amount of collagen tissue, degenerated Schwann cells, and cell debris. Degenerated Schwann cells were not encountered in young-adult mice. In the old group, end-plates of normal appearance occurred in the proportion of 40%. The young group had normally structured end-plates in the proportion of 85%. The lesions described are not specific for the aging process.

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INTRODUCTION

CORRELATIVE ULTRASTRUCTURAL studies have shown a structural heterogeneity in neuromuscular junctions of mammalian red, white, and intermediate skeletal muscle fibers (Murata and Ogata, 1969; Ogata and Murata, 1969; Shafiq *et al.*, 1969; Padykula and Gauthier, 1970; Duchen, 1971; Pulliam and April, 1979a; Galvas *et al.*, 1982). In the mouse and rat, and in man, the red (slow) muscle fibers are generally smaller than the white (fast) fibers, and the larger white fibers generally have larger regions of myoneural contact (Duchen, 1975). Previous studies have alluded to the functional implications of large axoplasmic surfaces in the motor end-plates as seen in white fibers *versus* the small points of axoplasmic contact in red and intermediate fibers (Padykula and Gauthier, 1970; Duchen, 1975). The appearance of the myoneural junction in fetal and neonatal rats has been described by Teräväinen (1968), while Zacks and Blumberg (1961), comparing the neuromuscular junctions of mice and man, pointed out the marked similarity between these two species.

To date, only a few studies have dealt with the effects of age on the fine structure of the

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motor end-plate in mammals. Most of the currently available ultrastructural studies have utilized muscles of young-adult animals. Fujisawa (1976) indicated structural changes in terminal axons of aged rats, based on three animals, and Tomonaga (1977) reported a reduction in the number of subsynaptic folds of different muscles from senile man. Recently, Cardasis (1983) described the neuromuscular junction of 11- and 26-month-old rat soleus and noted continued reorganization in this area. However, no detailed ultrastructural information is available on the Schwann cells and other cells populating the motor end-plate region of aging mammals. Recent experimental studies in our laboratory concluded that age per se exerts deleterious effects upon the structure of skeletal muscles (Ludatscher *et al.*, 1983; Silberman *et al.*, 1983).

MATERIALS AND METHODS

Six young and seven aged female C57BL/6J mice were used in this study. The mice in the young group were 6-months-old, and their weight was 20–25 g each; in the aged group the mice were 27-months-old, and their weight was 26–31 g each. All mice were killed by neck dislocation, and the gastrocnemius muscle was rapidly removed, cut into small pieces, and immediately immersed into a solution of 3% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, at 4°C for 2 hours. Specimens were then postfixed in 2% osmium tetroxide in 0.1 *M* cacodylate buffer for 1 hour, dehydrated through graded ethanols, and embedded in Epon. Semithin (1-μm) sections were cut from all the blocks, stained with 0.1% toluidine blue, and examined with the light microscope. The myelinated axons were easily identified on the semithin sections. Thin sections (600–800 Å) were cut semiserially from the above blocks, stained with uranyl acetate and lead citrate, and then examined with a Zeiss 9S electron microscope. Photographs of 48 motor end-plate regions containing 67 axon terminals from the group of young mice and of 60 motor end-plate regions containing 109 axon terminals from the group of aged mice were taken at various magnifications. The counting and the measurements of the axon terminals were done on micrographs showing longitudinal planes of similar magnification. The examination, the counting, and the measuring of all material were prepared by the same researcher.

RESULTS

Young adults

The motor end-plate region of many muscle fibers had an oval-shaped gutter which was occupied by a well-delineated axon terminal. The axonal cytoplasm had a normal appearance and contained mitochondria, presynaptic vesicles, a few tubular structures, and a few empty small vacuoles (Figure 1). The junctional folds were arranged at irregular distances from one another, and the secondary synaptic clefts were wide and less numerous (Figure 1). In most muscle fibers the axon terminal lay in an elongated gutter with the synaptic portion of the axonal membrane in close apposition to the sarcolemmal membrane (Figure 2). The postsynaptic sarcolemmic membrane forming the secondary clefts was ragularly and deeply infolded over a surface that varied in length, but that usually allowed for a large contact area as a result of the numerous infoldings. Often the secondary synaptic clefts had a "racquet-like" shape (Figure 2). A few collagen fibrils could be seen in the endomysial space. The axon terminal was covered by Schwann cell processes on the side that faced the endomysial space (Figures 1 and 2). The sarcoplasm in the sole-plate region contained ribosomal granules, tubules of endoplasmic reticulum, and mitochondria that varied in number from a few to small focal agglomerations. The sole-plate nucleus was normal sized and had a well-developed nucleus. Aberrations from the normal morphology were recorded in 15% of the total of 67 end plates studied (Table 1).

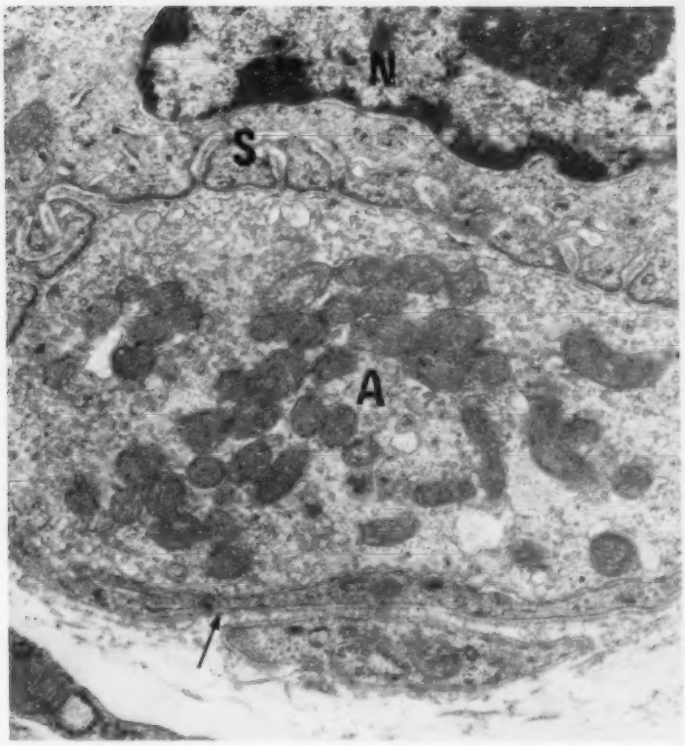

FIG. 1. Electron micrograph showing a motor end-plate of a 6-month-old mouse. The axon terminal (A) is oval-shaped and contains synaptic vesicles and a normal amount of mitochondria. The secondary synaptic clefts (S) are wide and relatively few. Arrow points to Schwann cell processes. N = sole plate nucleus. × 19,000.

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Aging animals

Motor end-plates were found in muscle fibers which appeared little or moderately degenerated. Some fibers were normal sized and some were atrophic. Large bundles of myelinated axons broke up, and the axons together with the Schwann cells approached the muscle fiber (Figure 3). In the region of the motor end-plate a single axon lost its myelin sheath and further branched into three or four axon terminals (Figure 3). The endomysial space was filled with a large amount of collagen fibrils and with Schwann cell processes (Figure 3). Normally structured end-plates similar to those described in the young group were also seen, in proportion of 40%. The morphological changes varied in kind and intensity from one motor end-plate to another in the same animal. The main changes are summarized in Table 1.

Primary gutter and axon terminals. In the end-plates of normal morphology the primary gutter was often egg-shaped and of normal size (Figure 4). Sometimes the gutter was small (Figure 5) or very small and irregularly shaped (Figure 6). The axon terminals occu-

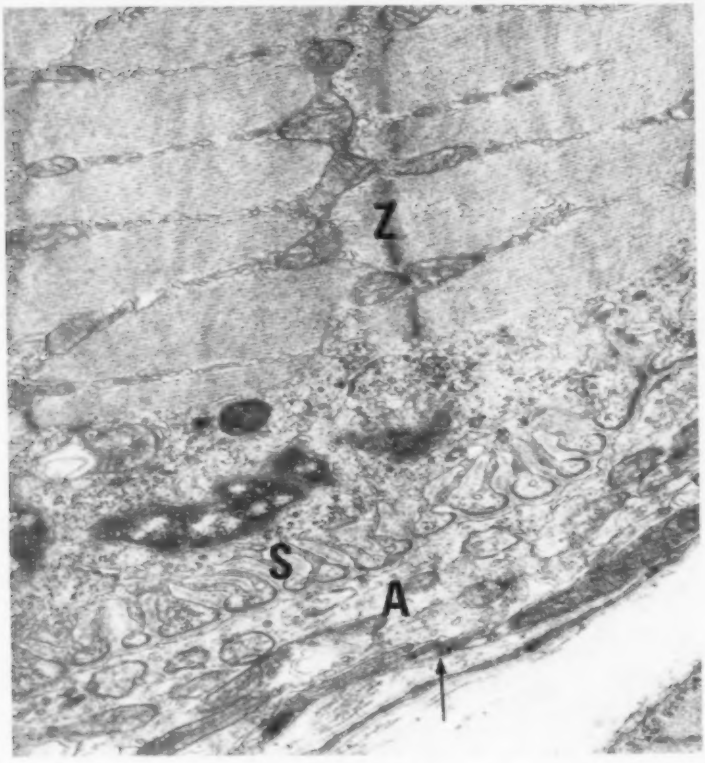

FIG. 2. Six-month-old mouse. An elongated axon terminal (A) has a large myoneural contact area. The secondary synaptic clefts (S) are numerous and have a "racquet-like" shape. The adjacent muscle is normal structured and shows paired mitochondria on each side of the Z-band (Z). Arrow points to Schwann cell processes. × 19,000.

TABLE 1. ULTRASTRUCTURAL EVALUATION OF MOTOR END-PLATES OF YOUNG AND AGING MICE

	6-months-old (No.)	*27-months-old (No.)*
Motor end-plate regions studied	48	60
Axon terminals counted in above regions	67	109
Regenerated axon terminals	3	11
Axon terminals showing "emptiness"	4	21
Axon terminals showing dense material and membranous bodies	2	13
Axon terminals showing marked shrinkage	0	7
End-plates showing marked abnormalities of junctional folds	1	12
End-plate regions showing marked degeneration of Schwann cells and/or their processes	0	22
Proportion of normally structured end-plates (%)	(85)	(40)

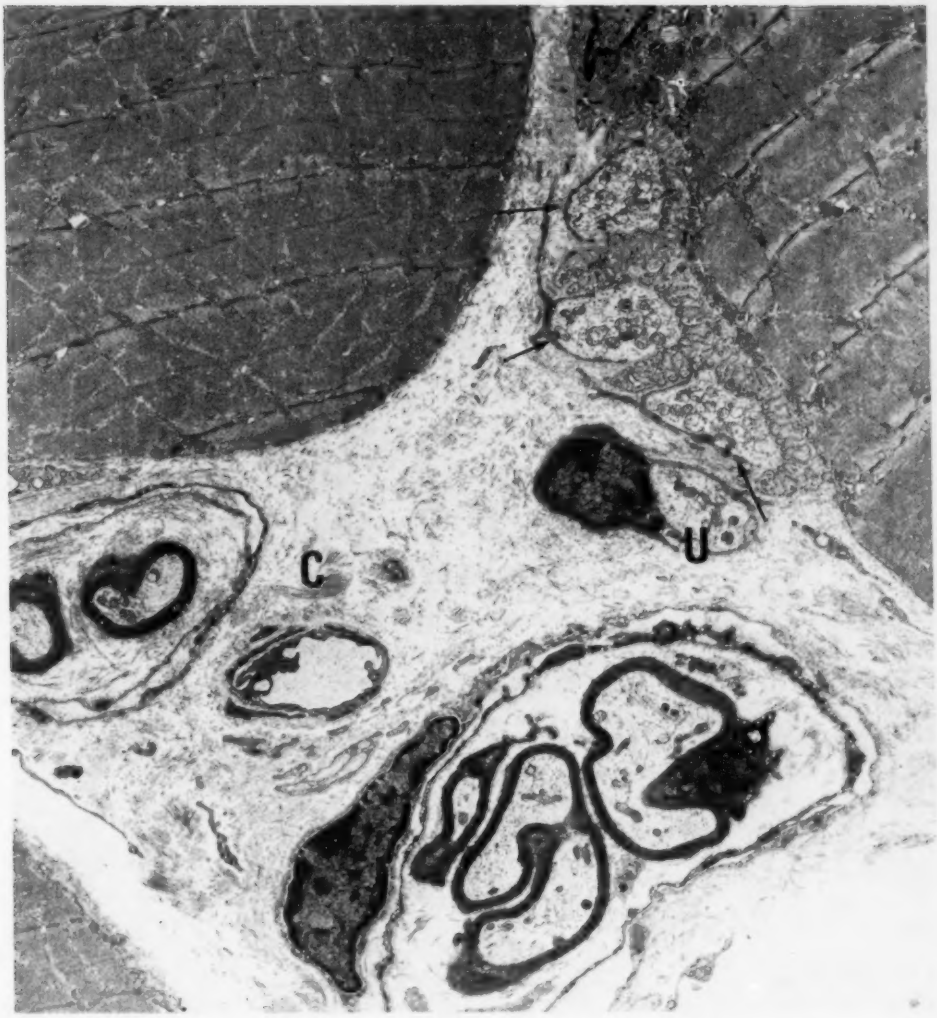

FIG. 3. Mouse B, 27-months-old. Two normal muscle fibers are seen on the upper part of the micrograph, and two bundles of myelinated axons and their Schwann cells appear in the lower part. An unmyelinated axon (U) is seen in close proximity to the muscle fiber on the right. The motor end-plate includes 3 normal axon terminals (arrow). The endomysial space contains multiple collagen fibrils (C). × 5,700.

pying the gutter showed a great diversity of alterations. In about 20% of the axons, the axoplasm appeared partly "empty" because of loss of mitrochondria and paucity of synaptic vesicles. Distended empty spaces which were irregularly bounded by a single membrane were encountered. Dark, homogeneous bodies or membranous bodies of various size and density were present (Figure 4). In a few animals the axon terminals appeared shrunken and contained dark, granular material that was condensed into irregular conglomerates (Figure 5). Dark and wide Schwann cell processes capped the degenerated

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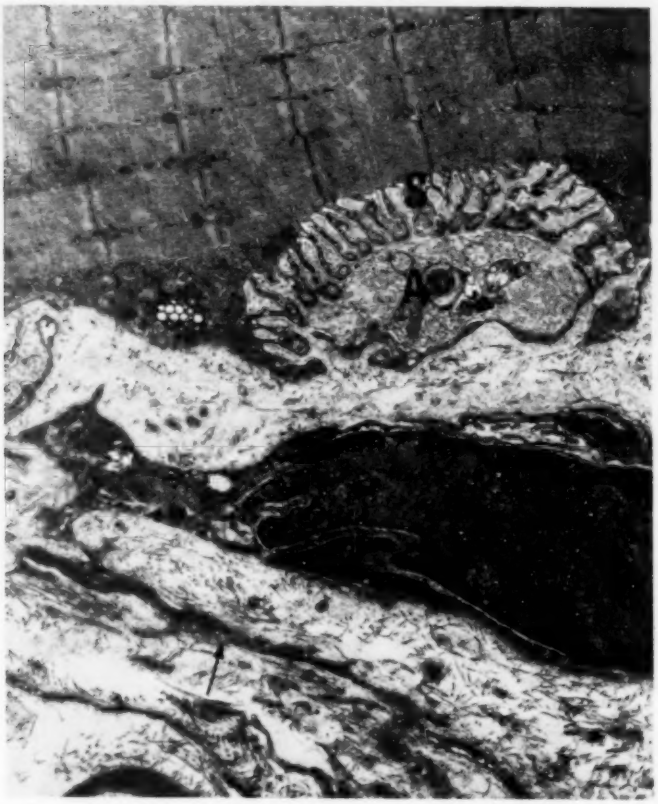

FIG. 4. Mouse C, age 27 months. The axon terminal shows dark membranous bodies (A). The secondary synaptic clefts are numerous and thin and closely arranged (S). The motor end-plate region is packed with collagen fibrils and Schwann cell processes (arrow). A degenerated Schwann cell containing a large, dense nucleus is seen in the center of the picture. × 9,800.

axon on the side facing the endomysial space (Figure 5). The shrunken axon terminal was sometimes distorted in shape, contained few synaptic vesicles, and lacked mitochondria, thus making it difficult to recognize the axon (Figure 6). In these cases the axon terminals were almost totally obstructed by a large amount of adjacent collagen tissue and cell debris from the motor end-plate region (Figure 6).

Secondary synaptic clefts. In many instances the junctional folds were evenly spaced and showed no abnormalities. Arborization of deep junctional folds was noted. Sometimes the folds were thinner than normal. Partial loss, distortion, and fragmentation of secondary synpatic clefts were seen in cases with severely degenerated axon terminals, making it impossible, therefore, to count or to measure the folds (Figures 5 and 6). Regions of exposed junctional folds were evident, and this resulted in a decrease in the effective area of synaptic contact (Figure 6). Total absence of secondary synaptic clefts was encountered in regions with axon terminal regeneration (Figure 7).

FIG. 5. Mouse D, age 27 months. The axon terminal contains conglomerates of dense, degenerated material (D). The axonal membrane facing the endomysial space is thick and is covered by a degenerated cell process (arrow). A macrophage (m) and dark cell processes are seen in close proximity to the motor end-plate. × 9,800.

Sole-plate. Sarcoplasmic strands interdigitated with the junctional folds and contained ribosomal granules and a few postsynaptic vesicles. The adjacent sarcoplasm contained ribosomes, mitochondria, and tubules of endoplasmic reticulum in varying number. Occasionally, loss of cristae was seen in degenerated mitochondria (Figure 6). Sometimes aggregates of parallel-oriented tubules were noted. The sole-plate nucleus was often moderately or strongly indented. Numerous lipofuscin granules of varying size usually occupied a significant portion of the sole-plate (Figure 6). Frequently, the lipofuscin bodies reached a diameter of 5 μm.

Endomysial motor end-plate region. The endomysial space contained a large amount of collagen fibrils, Schwann cells, and cell processes (Figures 4–6). The Schwann cells and their processes showed degeneration, and the limiting membrane was thick and denser than normal (Figure 5). Macrophages and fibroblasts were common (Figure 5). The degenerated Schwann cell cytoplasm appeared very dense and featured myelin figures (Figure 7). The delineating basement membrane-like material was fuzzy and dispersed in the area. The collagen tissue frequently obstructed the endomysial space and adhered to the sarcolemmic membrane and to the axonal membranes (Figure 6). Sometimes the collagen fibrils were embedded in a diffuse matrix substance of low density, which was similar in density to the basement membrane material.

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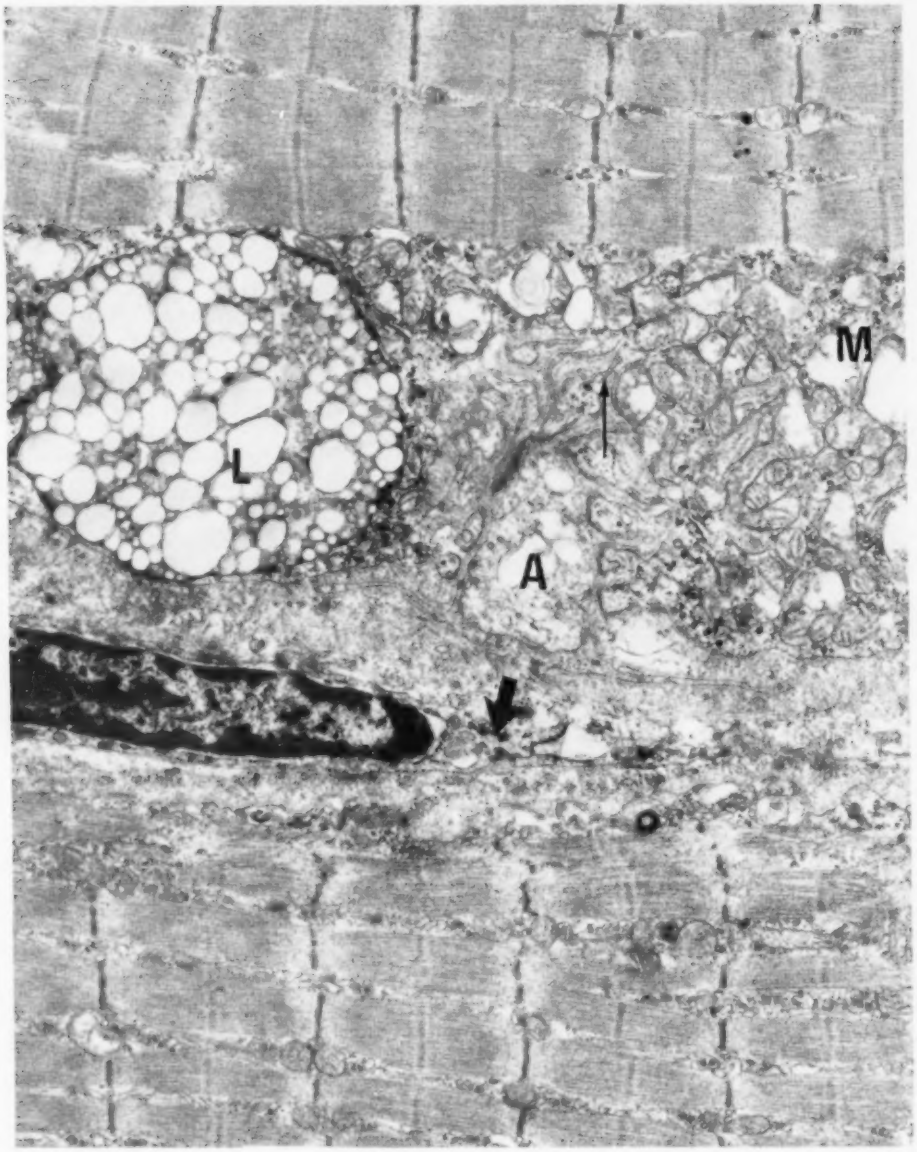

FIG. 6. Mouse E, age 27 months. A "shrunken" axon terminal (A) is devoid of mitochondria and of synaptic vesicles and shows enlarged empty vacuoles. The secondary synaptic clefts are few in number and are much distorted (arrow), and the area of synaptic contact is reduced in size. Degeneration and loss of cristae in the sole-plate mitochondria at M. Large fatty bodies at L. The endomysial space contains fuzzy basement membrane-like material and markedly degenerated cells (thick arrow). × 14,700.

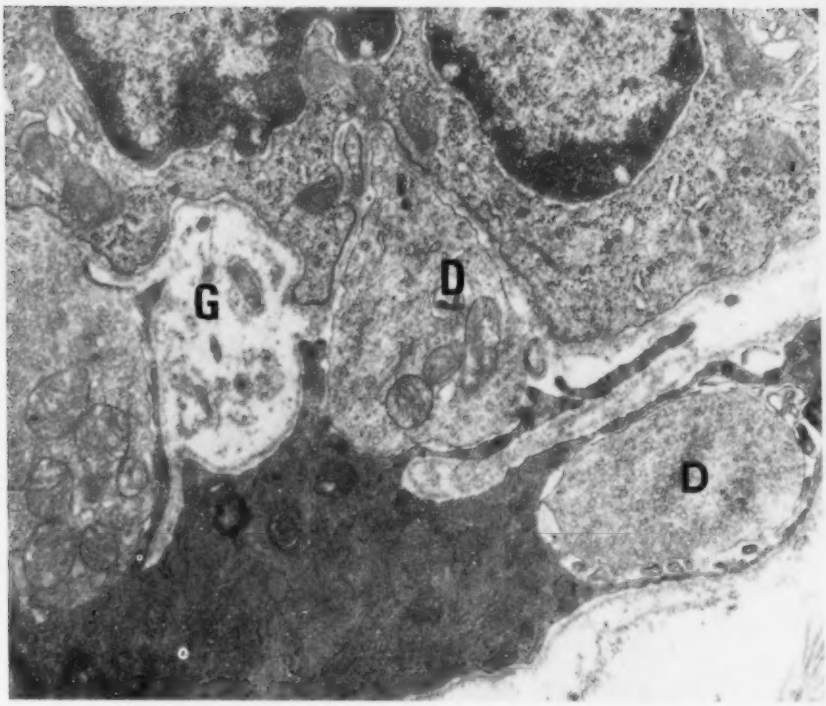

FIG. 7. Mouse F, age 27 months. This micrograph shows nerve terminal regeneration. Three axon terminals contain numerous synaptic vesicles and a few mitochondria (D). The adjacent gutter (G) contains granular material. The sole-plate region above is devoid of secondary synaptic clefts. At bottom of picture a strikingly degenerated Schwann cell appears completely dark and exhibits multiple myelin figures. × 19,000.

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DISCUSSION

In the present study no attempt was made to classify the end-plates into those of white and red or intermediate muscle fibers, due to the fact that in many instances the motor end-plates were severely altered and, therefore, difficult to type. The problem of detecting nerve endings in pathological muscles has been emphasized by Fardeau (1973), and we had similar difficulties concerning the aging mice. However, taking into consideration the length and the number of the secondary synaptic clefts, and the width of the Z-band, as suggested by others (Padykula and Gauthier, 1970; Duchen, 1971), we presume that most motor end-plates were pertaining to the white muscle fibers. This agrees with the general knowledge that the main part of the mouse gastrocnemius is a fast (white) muscle, with some fibers of intermediate and red type (Duchen, 1971).

In our material, slight variation in the morphology of individual end-plates was noted in the young mice. In comparison, the variation was striking in the group of aged animals, where the gastrocnemius of each mouse showed normally structured or little-affected end-plates and also severely altered ones. At this point we are unable to explain the factors responsible for the great diversity of lesions in aging animals. However, it is known from

experimental work that varied responses are achieved in terminals of different types of muscle fibers. Pulliam and April (1979*b*) noted that the severity of degeneration in the neuromuscular junction of the diaphragm after severance of the phrenic nerve varies according to the fiber type: the white fibers being most severely affected. A damaging effect of the tetanus toxin is recorded in the end-plates of the mouse soleus, which is a slow muscle; less damage is seen in the gastrocnemius, which is a fast muscle (Duchen, 1973). In denervation there is a preferential alteration of white fibers (Gauthier and Dunn, 1973). In the dystrophic mutant mouse, motor end-plates have been found in "healthy" fibers and in the best-preserved regions of the necrotic fibers, as well as in the most seriously damaged regions (Ontell and Haller, 1980).

In aging 27-month-old mice the lesions in the motor end-plates appear to be closely related to the increased amount of endomysial collagen fibrils and to cell debris containing degenerated Schwann cells, fibroblasts, macrophages, and cell process. The collagen tissue obstructing the endomysial space seems to compress, at the same time, the axon terminals, the sarcolemmic membrane, and the teloglial processes of the area. Proliferation of endomysial and perimysial collagen fibrils is a known phenomenon in aged mammals (Fujisawa, 1975; Ludatscher *et al.*, 1983). In our study it was not always possible to locate the end-plates from muscle fibers with the maximal damage. Most altered end-plates occurred in normal or moderately affected muscle fibers, or in fibers displaying tubular aggregates (Ludatscher *et al.*, 1983). The process of axonal regeneration was more conspicuous in aging animals.

The lesions described in this study are not specific for old age; since major alterations, exemplified by axonal shrinkage, axonal emptiness, and abnormalities of secondary synaptic folds, have been noticed also after administration of tetanus toxin (Duchen, 1973) in the dystrophic mutant mouse (Ontell and Haller, 1980) and in corticosteroid-induced masseter myopathy (Silbermann *et al.*, 1980). Dense granular masses and membranous bodies similar to those described in the axon terminals of some of our old animals have been recorded in denervation (Duchen, 1975). Many of the structural aspects in the aging mice correspond to those noticed in humans with myasthenic syndrome (Bickerstaff *et al.*, 1960; Woolf, 1966; Fardeau, 1973) and in experimentally induced myasthenia (Thornell *et al.*, 1976).

This study clearly reveals the morphological differences between the neuromuscular junctions of young-adult mice and those of aging mice. The most obvious difference is exemplified by the consistency with which degeneration of Schwann cells and their processes occurs in 27-month-old mice, while no degeneration is noticed in young adults. It has been reported that in aging rat soleus neuromuscular junction, degeneration and regeneration occur side by side (Cardasis, 1983). The decrease in the effective area of synaptic contact is the most important sign that suggests degeneration (Cardasis, 1983).

Our data indicate that besides damage to the area of synaptic contact, the most frequent sign of degeneration is registered at the level of the Schwann cell and its processes. The possibility arises that the development of changes in the primary cleft structures is dependent upon degeneration of Schwann cells, with a sequence of events similar to that experienced in denervation (Pulliam and April, 1979*a*).

According to our material, the weight of the animal is not an adequate indication of age, since no sensible difference was noticed in the weight of young-adult and old mice.

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AGE CHANGES IN DENSITY OF ENDOMETRIAL STROMAL CELLS OF THE RAT

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Abstract—Stromal cells were counted in the endometrium of the rat during aging. Cell counts were made in the subepithelial, middle and deep regions of the endometrium in the left uterine horn of virgin Sprague-Dawley rats at 36 days of age and at 4, 10, and 20–24 months. In all regions of the endometrium the density of stromal cells decreased with aging. Decrease in cell numbers per unit area was greatest in the middle and deep endometrium. In 20 to 24-month-old rats, the mean number of cell profiles within 0.085 mm² of the middle endometrium had decreased to less than one-third the number that was present within the same area in the 36-day animal. In the deep endometrium of these oldest rats, the mean number of cells had decreased to less than one-fourth. An age-associated decrease in density of stromal cells may contribute to alterations that have been observed in hormone-dependent uterine synthetic activities during aging.

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INTRODUCTION

DURING AGING in the rat (Wolfe *et al.*, 1942) and mouse (Loeb *et al.*, 1939), the stroma of the uterus, particularly in the endometrium, becomes more fibrous and less cellular as collagen accumulates. In the rat the endometrium is most cellular in the 6-day-old animal, and cell density decreases thereafter. This decrease in the number of cells does not occur uniformly. Collagen is deposited most abundantly in the peripheral endometrium, causing the cells in the outer region to become more widely separated from one another; on the other hand, the subepithelial stroma remains more cellular (Wolfe *et al.*, 1942). Distribution of endometrial stromal cells changes similarly in the aging mouse (Loeb *et al.*, 1939) and hamster (Rolle and Charipper, 1949). The relative extent of stromal cell decrease in the endometrium of the aged animal has not been investigated. Stromal cells synthesize the collagen of the endometrium. Among the cell types of the uterus, stromal and epithelial cells are the most responsive to ovarian hormones. Furthermore, stromal cells are more numerous and during implantation, undergo decidualization. Alterations in their number may be significant in studies of changes in uterine function during aging. The present study examines the change in density of endometrial stromal cells of the rat with increasing age.

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MATERIALS AND METHODS

Animals

The left uterine horn of 24 virgin Sprague–Dawley rats was studied. The animals, some of which were used in earlier studies from this laboratory on age changes in rat uterus, were of four ages. Groups of rats, (eight 36-day, three 4-month, four 10-month and eight 20 to 24-month-old animals) were maintained at controlled temperature and on a 14:10 h light/dark cycle. They were provided food and water *ad libitum*. All animals were ovariectomized 13 days before they were killed for study. Five days after ovariectomy, the rats were subjected to a series of daily injections of ovarian hormones on a schedule to support artificial induction of the decidual reaction (Craig, 1981; Craig and Jollie, 1981). Decidualization was induced in the right uterine horn by intraluminal injection of sesame oil, 0.1 ml. The left uterine horn served as the nondecidual control. Immature rats of this study were not included in earlier reports. They each received eight daily injections of progesterone, (1 mg) and on day 4 of progesterone treatment, subsequently, a single injection of estradiol 17-β, (.03 μg). On the ninth day after the beginning of hormone injections, uteri were removed, and trimmed of fat and connective tissue and processed for study.

Tissue preparation

Transverse tissue blocks from three levels of the uterus; namely, tubal, middle, and cervical, were processed for microscopy. For routine light microscopy, samples were fixed in Zenker's fluid, dehydrated in ethanol and embedded in Paraplast. Sections were cut at 4 μm and stained with hematoxylin and eosin. Tissues also were processed for embedment in Durcupan ACM as described previously (Craig, 1981). One micrometer sections of Durcupan-embedded tissues were collected on slides and stained with toluidine blue (Lynn *et al.*, 1966).

Cell counting

Cells were counted on a Wild M 20 binocular microscope at × 600 with the aid of an eyepiece graticule calibrated against a slide micrometer. Counts were made on nine sections of the uterus from each animal, three sections from each uterine level. To avoid counting the same cell more than once, sections on which counts were made were no less than 200 μm apart. On each uterine section cells were counted in the seven regions that are illustrated in Figure 1. In each section within each region, 3–5 individual cell counts were made in fields of .085 mm^2. The mean of the total individual cell counts in each case was used as the number of cells per field for each region. With the exception of avoiding fields, which included glands, field choice was random. Stromal cells are stellate, and their processes extend in different directions from the cell body. Therefore, in order to avoid counting small parts of cells, within any field of measurement cells were counted only if their nucleus could be seen. Cells that extended partially into the field of observation were counted only if more than one-half of the profile of the nucleus was within the limit of the field of count. The results are expressed as the mean and the standard error of the mean (SEM).

Data analysis

Statistical significance between cell counts in the endometrium of rats of different age groups was analyzed by Student's t test.

RESULTS

Results of the cell count appear in bar-graph form in Figure 2. As illustrated, with aging, endometrial stromal cells decreased in density in all regions where cell counts were made. The differences between the mean numbers of stromal cells per unit area in the younger (36-day, 4- and 10-month-old) animals and the 20 to 24-month-old rats were statistically significant ($P < 0.001$ for all regions of all ages except region 7 of 10-month/20 to 24-month animals where $P < 0.005$). The number of cells per .085 mm^2 decreased progressively, and to a greater extent, in the middle and deep endometrium. By the age of 20–24 months, the mean number of cell profiles per unit area of tissue section in the middle region (regions 2 and 5 in Figure 1) had decreased to less than one-third of the number present in the 36-day-old rat, while the number of cells was less than one-fourth of 36-day-old animals in the deep endometrium (regions 3 and 6 on Figure 1). The reduction in the density of cells was somewhat less in the subepithelial region. Except in the

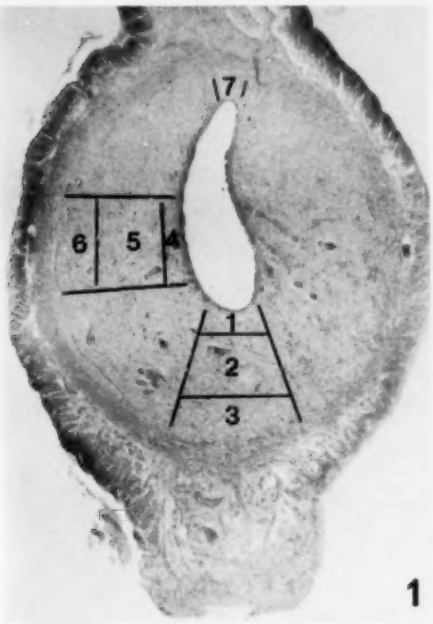

FIG. 1. Cross-section of the uterine horn of a 10-month-old rat. Stromal cells were counted in the 7 outlined and numbered areas of the endometrium. × 34

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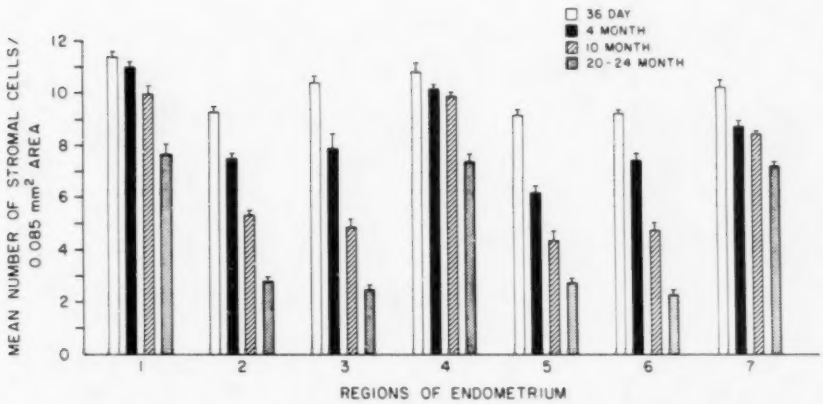

FIG. 2. Changes with aging in number of cells in different regions of the endometrium. Area numbers in the figure correspond with regions of the endometrium as illustrated in Figure 1. Each bar represents the mean and the vertical line ± SEM for the group of rats of each age.

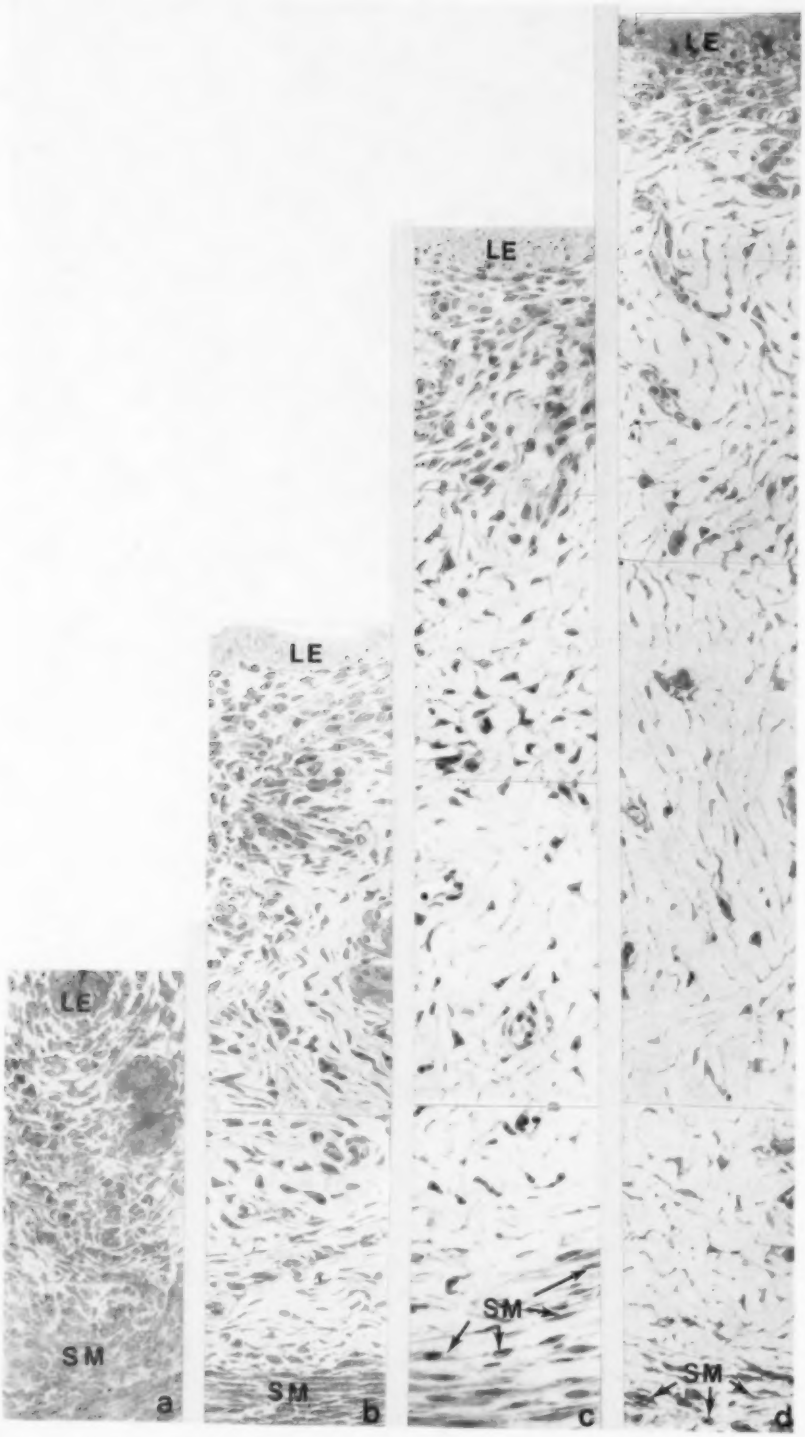

FIG. 3. Sections of antimesometrial endometrium of rats of ages 36 days (a), 4 months (b), 10 months (c), and 20–24 months (d). The micrographs illustrate the changes in cell density and distribution that typically occur in this region of the endometrium in aging. Luminal epithelium (LE); Smooth muscle (SM) × 224

mesometrial area (region 7, Figure 1), the reduction occurred primarily between 10- and 20–24 months of age. These changes in cell density and distribution are shown in Figure 3.

DISCUSSION

Results presented here extend the early observations of Burack *et al.* (1941) and Wolfe *et al.* (1942). The present data demonstrate more precisely the relative decrease in density of stromal cells in different regions of the endometrium of the rat during aging. The use in this study of 4 μm paraplast and 1 μm Durcupan sections for the cell counting prevented an overlap of cells due to section thickness. Numerous cell types (i.e., cells other than the fibroblast-like "stromal" cell) can be found in the stroma of the uterus. Thinness of the sections therefore, also permitted a more accurate identification of stromal cell type.

Saiduddin and Zassenhaus (1979) reported that there is less DNA per mg of total protein in the uterus of the aged rat when compared with the young. Their findings support biochemically the histologic observations presented here that there is a reduction in cellularity in the uterus during aging. Uterine growth parallels body growth up to a point, largely by deposition of collagen. Consequently, our results demonstrate the extent of decrease in stromal cell number within a given area, not necessarily a decrease in absolute cell number.

In terms of relative thickness, endometrium makes up the greater part of the wall of the uterus in the young rat, and its proportion becomes greater as the uterus increases in size during aging (Wolfe *et al.*, 1942). Increase in the mass of endometrium alone, or of the uterus without comparable increase in number of parenchymal cells, would influence results of biochemical studies where cellular responses of the aged uterus are compared with those of the young animal when either tissue weight or tissue volume is a principal parameter in the study. An age-associated decrease in density of stromal cells may contribute to alterations that have been observed in hormone-dependent uterine synthetic activities during aging.

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EFFECTS OF AGING ON THE LIPID ORDER AND COMPOSITION OF RAT ADIPOCYTE GHOSTS

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Abstract—An analysis of the cholesterol/phospholipid ratio of adipocyte ghosts from rat epididymal fat pads shows a significant increase with age ($P < 0.005$). An attempt to correlate these changes with the order of the lipid matrix was made using the stearic acid spin label 2-(3-carboxypropyl)-4, 4-dimethyl-2-tridecyl-3-oxazolidinyloxyl [I(12,3)]. Although order was negatively correlated with temperature in preparations from both 6- and 24-month-old rats, no effect of age could be detected.

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INTRODUCTION

IN RECENT years much interest has focused on changes in membrane fluidity, order and mobility during aging (Armbrecht *et al.*, 1982; Rivnay *et al.*, 1980; Woda *et al.*, 1979; Hershkowitz *et al.*, 1982; Rivnay *et al.*, 1979; Dave and Knazek, 1983). With few exceptions (Armbrecht *et al.*, 1982; Hamm *et al.*, 1982; Butterfield *et al.*, 1982), most such investigations have reported decreased fluidity with increasing age.

Although many factors may contribute to reduced membrane fluidity (Quinn, 1981; Shinitzky and Inbar, 1976), the majority of aging studies implicate increases in cholesterol to phospholipid rations (Araki and Rifkind, 1980; Birnbaum and Baird, 1978; Hegner and Platt, 1975; Rivnay *et al.*, 1980; Dave and Knazek, 1983). In fact, cholesterol to phospholipid ratios have been reported to increase with age almost universally, even in cases where no loss of fluidity could be detected (Armbrecht *et al.*, 1982; Birnbaum and Baird, 1978). The consequences of age-related changes in such membrane physiochemical properties include altered structure and function, and most notably, impaired responsiveness to many hormones, neurotransmiters and related agents (Crews *et al.*, 1981; Hershkowitz *et al.*, 1982).

The glucose transport system of the rat adipocyte plasma membrane exhibits a generalized increased resistance to regulation by various agents as age increases (Roth and Livingston, 1979). Although this type of impairment is apparently due to receptor related changes in some cases (Roth and Livingston, 1976; Roth and Hess, 1982), other effects of

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age seem to be intrinsic to the plasma membrane itself (Roth and Hess, 1982). In fact, adipocytes exhibit a progressive increase in the percentage of oxidized sulfur groups on the cell membrane proteins during aging (Hughes *et al.*, 1980). Since the rat adipocyte ghost is a well characterized model for examining altered membrane regulation of hormone action (Sauerheber *et al.*, 1980; Cooper and Gregerman, 1976; Murray *et al.*, 1979), it was considered ideal for investigation of the role of membrane dynamics in altering cellular function during aging.

In the present study we have thus investigated the effects of aging on the order and lipid composition of the rat adipocyte ghost. Although ghosts contain internal as well as plasma membranes (Rodbell, 1967), this system was considered more suitable for an aging study than the purified plasma membrane, which is known to lose various functional activities during preparation (Carter *et al.*, 1971; Kissebah *et al.*, 1975; Kelly *et al.*, 1984.) Lipid order has been assessed by various methods, which measure different aspects of membrane fluidity and order. These include electron spin resonance fluorescence and nuclear magnetic resonance.

Electron spin resonance of stearic acid labelled ghosts was chosen for our purposes, since this method is able to distinguish between different pools of lipid which differ considerably in mobility (Jost *et al.*, 1973). Furthermore, a recent study by Armbrecht *et al.*, (1982) has used this method on a preparation of hepatic microsomes, which are compositionally somewhat analagous to adipocyte ghosts in that they contain internal membrane components. The unusual finding in this system of an increase in fluidity during aging suggested the importance of pursuing similar studies on other complex systems.

MATERIALS AND METHODS

Male virgin Wistar rats, aged 6- and 24-months were obtained from the colony of the Gerontology Research Center, NIA (NIH), Baltimore, MD. This colony is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Rats were maintained in the NIH Purina chow diet *ad libitum*, and were on a 0600-1800 h lighting schedule. Body weights were 500–600 g for both groups.

For preparation of adipocytes, animals were killed by cervical dislocation between 0700 and 0900 h and the epididymal fat pads rapidly removed into saline warmed to 37°C. Isolated fat cells were then prepared by treatment with collagenase (Type I, Worthington Bichemical Corp.) by the method of Rodbell (1964). Adipocyte ghosts were also prepared by the method of Rodbell (1967). Protein concentrations were determined by the method of Lowry *et al.* (1951).

Lipids were extracted by chloroform-methanol 2:1 (v/v) and filtered through Schleicher and Schuell prepleated filters. The original tubes and the filters were rinsed four times with additional solvent. The extracts were evaporated to dryness in a heated water bath while being flushed with nitrogen. Samples were redissolved, in chloroform-methanol 2:1(v/v) and transferred volumetrically to teflon-lined screwcapped tubes which contained 20% v/v 0.58% NaCl for the washing procedure according to Folch *et al.* (1957). The separated lower phases were evaporated to dryness in a National Appliance Co. Vacuum Oven, Model 5831. Shortly after the addition of 1.0 ml chloroform: methanol, 2:1 (v/v) to each sample, aliquots for analysis were taken. This was done in a 4°C cold room to minimize evaporation of the solvent.

Analyses of total cholesterol were carried out according to Abell *et al.* (1952) in duplicate on aliquots evaporated to dryness in the vacuum oven. Two ml of modified Liebermann-Burchard reagent (Sigma, St. Louis), stored at −60°C were added to each sample with vigorous vortexing for 15–30 seconds. The absorbance at 620 nm was determined within 20–40 minutes. Standards were prepared from Sigma Cholesterol Stock Standard. Phosphorous analysis was carried out according to the procedure of Bartlett (1959) on duplicate or triplicate aliquots. All tubes were nitric acid-washed prior to the reaction. The Fiske and Subbarow reducer was obtained from Sigma, St. Louis, and KH_2PO_4 used for standards from Allied Chemical Co. The value of 25 g of phospholipid for each g of P_i was used in all calculations.

The stearic acid spin label 2-(3-carboxypropyl)-4, 4-dimethyl-2-tridecyl-3-oxazolidinyloxyl [I(12,3)] was obtained from Syva, Palo Alto. Prior to spin labelling, and immediately after preparation, the ghosts were adjusted to 0.6 m*M* ethylene diamine tetraacetic acid (EDTA), 18.6 μg/ml acetyl-2-leucyl-2-leucyl-L-arginal hemisulfate (Leupeptin), 1.2 m*M* phenyl methyl sulfuryl fluoride (PMSF) and 3000 KIU/ml of Trasylol. This

treatment was necessary to inhibit the proteolysis, which has been shown to occur with adipocyte ghosts (Rifkind *et al.*, 1982).

A 1.0 m*M* solution of the spin probe in ethanol was stored at −60°C. Aliquots of this solution were then transferred to a pear-shaped flask and dried with a stream of dry nitrogen. The samples of ghosts from 6-month or 24-month-old rats were then transferred to these flasks and labelled according to previously published procedures (Sauerheber *et al.*, 1977; Elgavish *et al.*, 1983).

In order to minimize possible changes due to storing of ghosts, the labelling and subsequent electron spin resonance analysis were performed immediately after preparation of ghosts, prior to determination of protein concentration. Therefore, the volume of spin probe was estimated on the basis of the ghost volume to provide a final probe concentration of approximately 3.8 μg/mg of membrane protein. It has been shown that at this concentration no probe-probe interactions are detected in the ESR spectrum (Sauerheber *et al.*, 1977). In our experiments, an ESR spectrum was measured immediately after labelling. If any probe interactions were detected, the sample was diluted with an additional aliquot of the same batch of adipocyte ghosts.

ESR spectra of the spin labelled adipocyte ghosts were measured on a Jeolco JES-ME-IX spectrometer equipped with a variable temperature accessory used to maintain a constant temperature to within ± 0.2°C. The actual temperature of the sample was determined by an Omega digital meter using a copper-constantin thermocouple.

RESULTS AND DISCUSSION

Lipid composition

Table 1 shows the phospholipid, cholesterol and cholesterol/phospholipid ratio (CL/PL) of adipocyte ghosts obtained from rats 6- and 24-months of age. The results indicate that the changes in total phospholipid and cholesterol with age are not significant. The errors in these values are particularly large because the cells must first be converted to ghosts prior to extraction and lipid determination. However, the changes in the CL/PL ratios which are not affected by loss of cells during sample preparation, show a very significant ($P < 0.005$) increase during aging.

The results of Table 1 show that the cholesterol to phospholipid ratio of adipocyte ghosts for rat epididymal fat pads increases with age. This observation is consistent with the findings of other laboratories for a number of different systems where a progressive increase in the CL/PL ratio has been reported (Birnbaum and Baird, 1978; Rivnay *et al.*, 1980; Araki and Rifkind, 1980).

Lipid order

Figure 1 shows typical electron spin resonance spectra of 6-month-old rat epididymal fat pad adipocyte ghosts labelled with I(12,3) at 7°C, 12°C, 19°C and 36°C. Indicated on

TABLE 1. EFFECT OF AGE ON LIPID COMPOSITION OF RAT ADIPOCYTE GHOSTS[a,b]

	Phospholipid (mg/ml of cells) × 10^3	*Cholesterol (mg/ml of cells) × 10^2*	*CL/PL mole/mole*
6 mo.	6.2 ± 2.5	1.3 ± 0.4	0.446 ± 0.026[c]
24 mo.	6.1 ± 2.5	1.6 ± 0.6	0.516 ± 0.041[c]

[a]Phospholipid and cholesterol were both determined on lipid extracts obtained from the ghosts. The CL/PL ratio was determined directly from these values. The volume of cells prior to ghost formation was used in determining the absolute phospholipid and cholesterol values.

[b]The values given in the table correspond to means ± standard deviations for five samples.

[c]The difference between the CL/PL ratios for young and old adipocyte ghosts is significant with $P < 0.005$ as determined by Student's t test.

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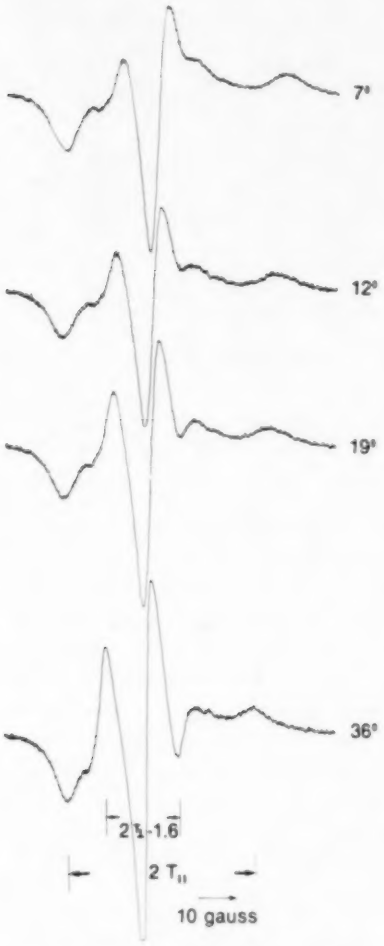

FIG. 1. Electron spin resonance spectra at various temperatures of adipocyte ghosts obtained from 6-month-old rat epididymal fat pads labelled with the stearic acid spin label [I (12,3)].

the 36°C spectra are the methods used to determine the hyperfine splittings $T_{\parallel}$ and $T_{\perp}$ necessary to calculate the order parameter (Sauerheber *et al.*, 1977; Elgavish *et al.*, 1973).

The precision necessary to determine the small changes in fluidity expected with age (Hegner *et al.*, 1979; Armbrecht *et al.*, 1982; Butterfield *et al.*, 1982) was obtained by running the low field and high field bands several times at increased gain (Sauerheber *et al.*, 1977). In this way it was possible to obtain values of $T_{\parallel}$ and $T_{\perp}$ for any sample with standard errors of less than 1%. It was noted that at low temperature it is essentially impossible to determine $T_{\perp}$ from the spectra. In order to extend our analysis of the temperature dependence of these spectra (Figure 2) to the range below 12°C, we have used $2T_{\parallel}$ as a measure of the order (Sauerheber *et al.*, 1977). A significant increase in $2T_{\parallel}$, corresponding to an increase in order is observed as the temperature decreases (Figures 1 and 2).

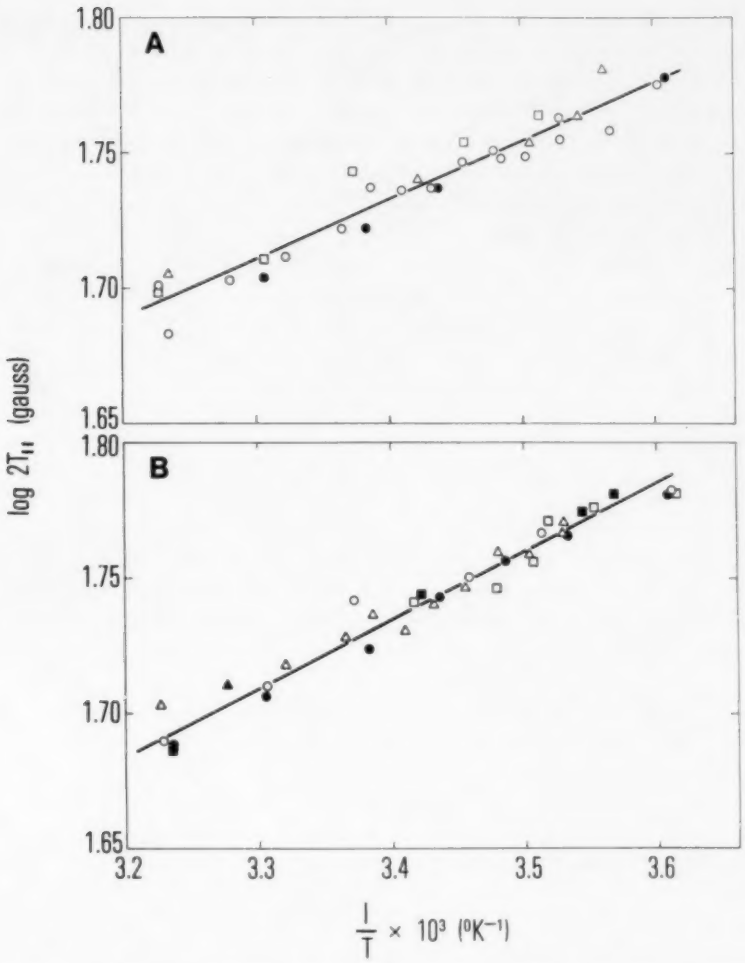

FIG. 2. Plot of log $2T_{11}$ versus the reciprocal temperature for adipocyte ghosts labelled with the stearic acid spin label I (12,3). Different symbols correspond to different experiments. The straight lines are the least squares linear regression for all the points. a—ghosts prepared from 6-month-old rats. Regression line log ($2T_{11}$) = 0.218 (reciprocal temperature) + 0.99. b—ghosts prepared from 24-month-old rats. Regression line log ($2T_{11}$) = 0.250 (reciprocal temperature) + 0.91.

However, analysis of variance revealed no significant age difference in the values of $2T_{11}$ or the change in $2T_{11}$ with respect to temperature. *F* values comparing Figure 2A and 2B with respect to elevations and slopes are 2.50 (df 1,63) and 1.43 (df 1,62), respectively.

The detected 16% increase in the CL/PL ratio found when comparing adipocytes from 6-month and 24-month-old rats is expected to produce more than a 6% increase in the electron spin resonance detected order parameters (Suda *et al.*, 1980). Such increases in lipid order have been detected in most of the cases where the CL/PL ratio increases during aging (Rivnay *et al.*, 1979, 1980). The expected change in lipid order associated with the

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observed change in lipid composition is well above the range of values for $2T_{11}$ found at any temperature (Figure 2) and should have produced significant changes in the ESR results. The failure to observe such change must thereby be attributed to other factors which compensate for the increase in the cholesterol content in these systems.

Several other exceptions in which the usual age associated increase in cholesterol content of lipid bilayers does not correspond to a decrease in fluidity (Armbrecht *et al.*, 1982; Butterfield *et al.*, 1982) have been reported. Particularly noteworthy are the results of Armbrecht *et al.* (1982), in which an increase in fluidity was reported in aged hepatic microsomes. This same group had earlier reported an increase in cholesterol content (Birnbaum and Baird, 1978) for this same system. They attributed the increase in fluidity to a change in the percent unsaturated fatty acids, which has also been shown to alter lipid fluidity (Shinitzky and Inbar, 1976).

An additional factor can, however, compensate for an increased cholesterol content in studies such as ours using intact ghosts containing internal membranes (Rodbell, 1967; McKeel and Jarett, 1970; Avruch *et al.*, 1976). Preliminary results from our laboratory indicate that these internal membranes have lower cholesterol levels than the plasma membrane. A change in the relative composition of these two lipid pools would thus change the overall lipid composition to a much greater extent than the ESR or other order and fluidity parameters. Certain important age associated changes in the organization of cell can thus perhaps only be detected using intact cells or ghosts. A thorough investigation of these two possibilities is beyond the scope of the present preliminary study and will be addressed in the future.

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AGE-RELATED DIFFERENCES IN FOOD INTAKE, BODY WEIGHT, AND SURVIVAL OF MALE F344 RATS IN 5°C COLD

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Abstract—Male F344 rats, 3, 12 and 21 months old, were acclimated at 5°C for 7 to 9 weeks. Controls were maintained at 23°C. Food intakes and body weights were monitored weekly. All animals in these age groups survived. However, 25-month-old rats placed in 5°C for 3 weeks had only a 46% survival. All age groups responded to 5°C exposure by different but always significant increases in food intake. Peak food intake was 100% above basal in the 3- and 21-month-olds and 80% above the basal in the 12-month-olds. These increases were sufficient to maintain body weights in the 3- and 12-month-olds, but in the 3-month-olds growth was retarded. The 100% increase of food intake in the 21-month-olds was not adequate to maintain body weight. The 25-month-old rats also increased their food intake significantly in cold, but large weight loss incurred in both the survivors and the non-survivors. Food intake and energy efficiency were different at different ages and under different environmental conditions in male F344 rats.

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INTRODUCTION

COLD ACCLIMATION studies on rodents have been performed primarily on young male Sprague-Dawley (S-D) or Wistar rats. Young male S-D rats acclimatize readily to a 5°C environment and when acclimated exhibit improved cold tolerance to a more severe cold challenge (Depocas *et al.* 1957). However, old (25 month) male S-D appeared to be vulnerable to cold—36% survival (5 out of 14) versus 100% survival of young (18 out of 18) in 3-week 5°C exposure and 100% in young and old S-D in 23°C (Kiang-Ulrich and Horvath, 1979). This finding imposes a serious restraint in the study of cold physiology in aging rats. Recently the Fischer 344 (F344) strain has become available for gerontological research. However, minimal information on the response of F344 rats to cold stress is available. Gambert and Barboriak (1982) reported that 14% of 6-week-old, 50% of 6-month-old, and 71% of 18-month-old male F344 rats survived 12 days 4–5°C exposure, and suggested increasing age might have improved the chances of survival in this strain, whereas younger animals were at great disadvantage. On the other hand, we found that

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In conducting this research, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee for Laboratory Animal Facilities and Care, of the Institute of Animal Laboratory Resources, National Academy of Sciences—National Research Council.

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3-to 4-month-old male F344 could be successfully cold acclimated (5 weeks in 5°C, 100% survival) similarly to young S-D (Kiang-Ulrich and Horvath, 1984). We now report on the age-related difference in survival of male F344 rats of 3, 12, 21, and 25 months exposed to either 5°C or 23°C.

MATERIALS AND METHODS

Male F344 rats were obtained from Charles River Breeding Laboratories, Wilmington, MA, and maintained in our vivarium conventionally, one per cage, with 12 h light and dark cycles. Water and Purina Lab Chow were provided *ad libitum*. Body weights and food intakes were monitored weekly. All were routinely monitored for signs and symptoms of diseases.

Group I consisted of 26 weanlings followed throughout their lives. Twelve of these were studied beginning at 3 months of age and another 12 at 12 months. During each period of study, half of each age group were placed in a 5°C environment (CA) for 7 or 9 weeks. The control rats remained in 23°C (non-CA) for the same period of time.

Group II consisted of 38 male retired breeders obtained at 20 months of age. According to the supplier, this colony had a median survival of 24 months. Twelve of these were studied beginning at 21 months of age. Six were exposed to 5°C and six to 23°C during the next 7 weeks. The remaining 26 rats were maintained for study beginning at 25 months of age. At that time 6 of the 26 animals had succumbed; 13 were placed in 5°C and 7 in 23°C. These rats were observed for only 3 weeks.

The differences in body weights and food intakes at 5°C and 23°C at different time intervals in each age period studied were statistically evaluated by analysis of variance and covariance with repeated measures across time. Where significant effects were found, tests of simple main effects (Tukey's) followed. The 0.05 level of significance was selected for rejection of the null hypothesis. Survival rates in either environmental condition were compared using the chi square test.

RESULTS

3-month-old rats

All animals in this age group survived the 7 weeks of 5°C exposure. An immediate and dramatic increase in food intake was observed in animals in the 5°C environment. Peak increase, 100% greater than basal levels measured at the beginning of the test, occurred within 3 weeks. During the same period the non-CA rats decreased their food intakes gradually and insignificantly over the same time period. Food intakes were significantly ($P < 0.001$) and consistently higher in the CA rats than in the non-CA. Body weights were significantly ($P < 0.001$) greater in the non-CA rats than in the CA rats in all time intervals. At the end of 7 weeks the non-CA rats had gained 46 g, while the body weight of the CA rats was not significantly changed during their cold exposure. When CA rats were removed from the cold at the end of 7 weeks, food intake decreased drastically but growth resumed (Figure 1).

12-month-old rats

All animals in this age group survived the 9-week test period. Animals in this age group had significantly ($P < 0.001$) greater mean body weights (376 g) and significantly ($P < 0.001$) smaller food intakes (52 g $\cdot$ kg^{-1} $\cdot$ day^{-1}) than 3-month-old rats. Growth of male F344 appeared to have stabilized, since the mean weight of non-CA rats at the end of 9 weeks was similar to that measured at the beginning of the test. Their food intake also remained constant from the beginning to the end. Upon exposure to 5°C, dramatic increases in food intake occurred immediately. A peak increase of 80% over the basal level occurred within 4 weeks. Food intake was sufficient to maintain body weight, as there was no sig-

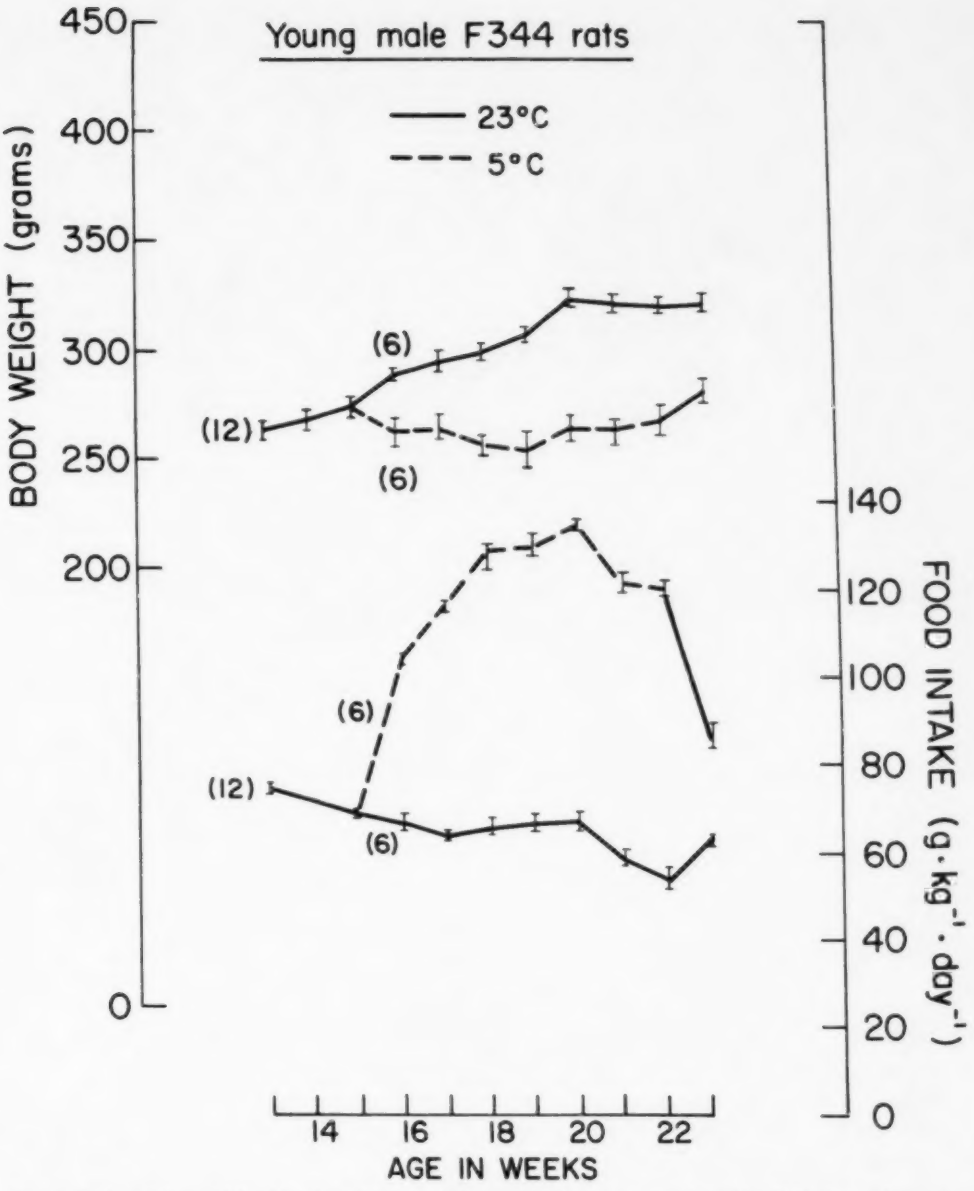

FIG. 1. Body weights and food intakes of 3-month-old male F344 rats measured weekly. Each data point represents mean ± SE. Number of animals in parentheses. The 7-week period of 5°C exposure began at week 15 and ended at week 22. These animals were then returned to 23°C.

nificant difference in body weight over the 9-week test period. There was no significant difference in body weight between the CA and non-CA groups at any time during the test period, although the food intakes were significantly ($P < 0.001$) different between the two groups at all times (Figure 2).

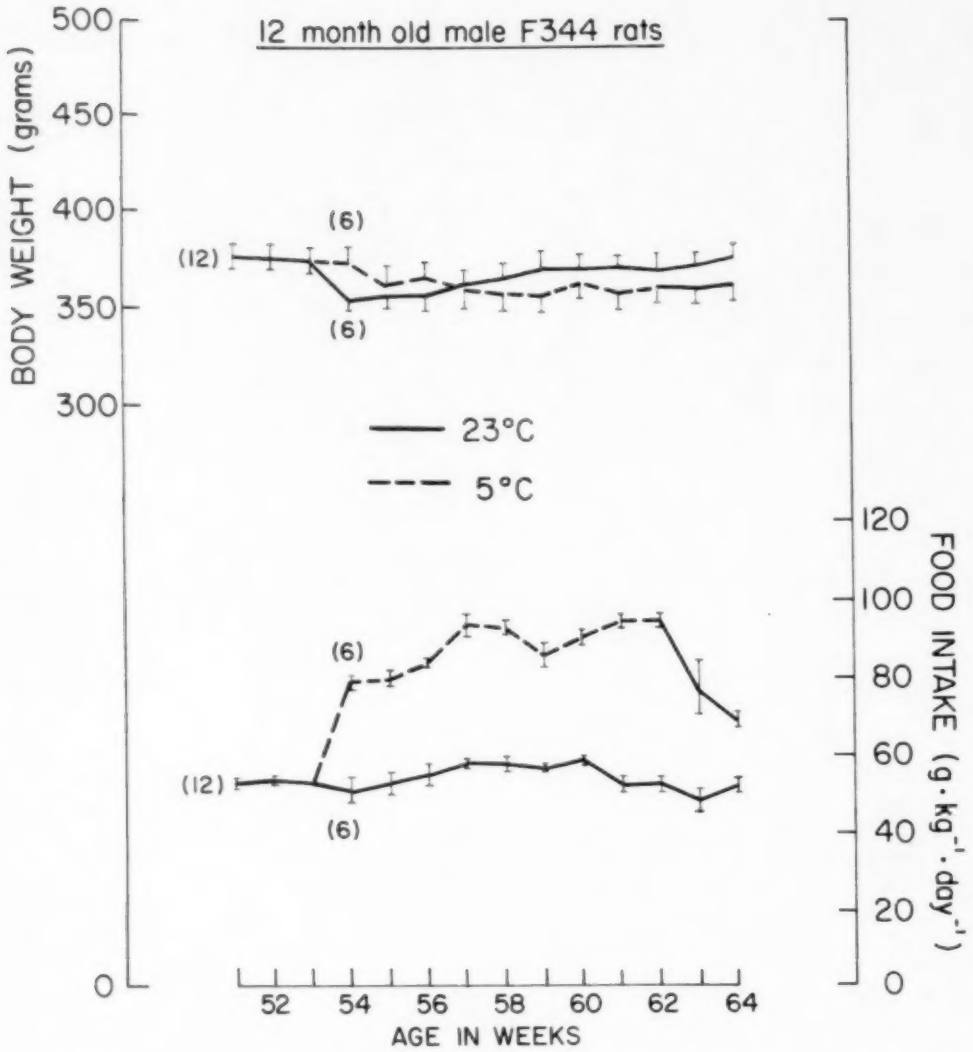

FIG. 2. Body weights and food intakes of 12-month-old male F344 rats measured weekly. Each data point represents mean ± SE. Number of animals in parentheses. The 9-week period of 5°C exposure began at week 53 and ended at week 62. These animals were then returned to 23°C.

21-month-old rats

All rats in this age group survived the 7-week test period. In the non-CA rats there was no significant difference between the body weights measured at the beginning of the test period and the weight at the end of 7 weeks. The CA animals progressively lost weight and the difference was significant after 4 weeks ($P < 0.01$) as well as at 7 weeks ($P < 0.01$).

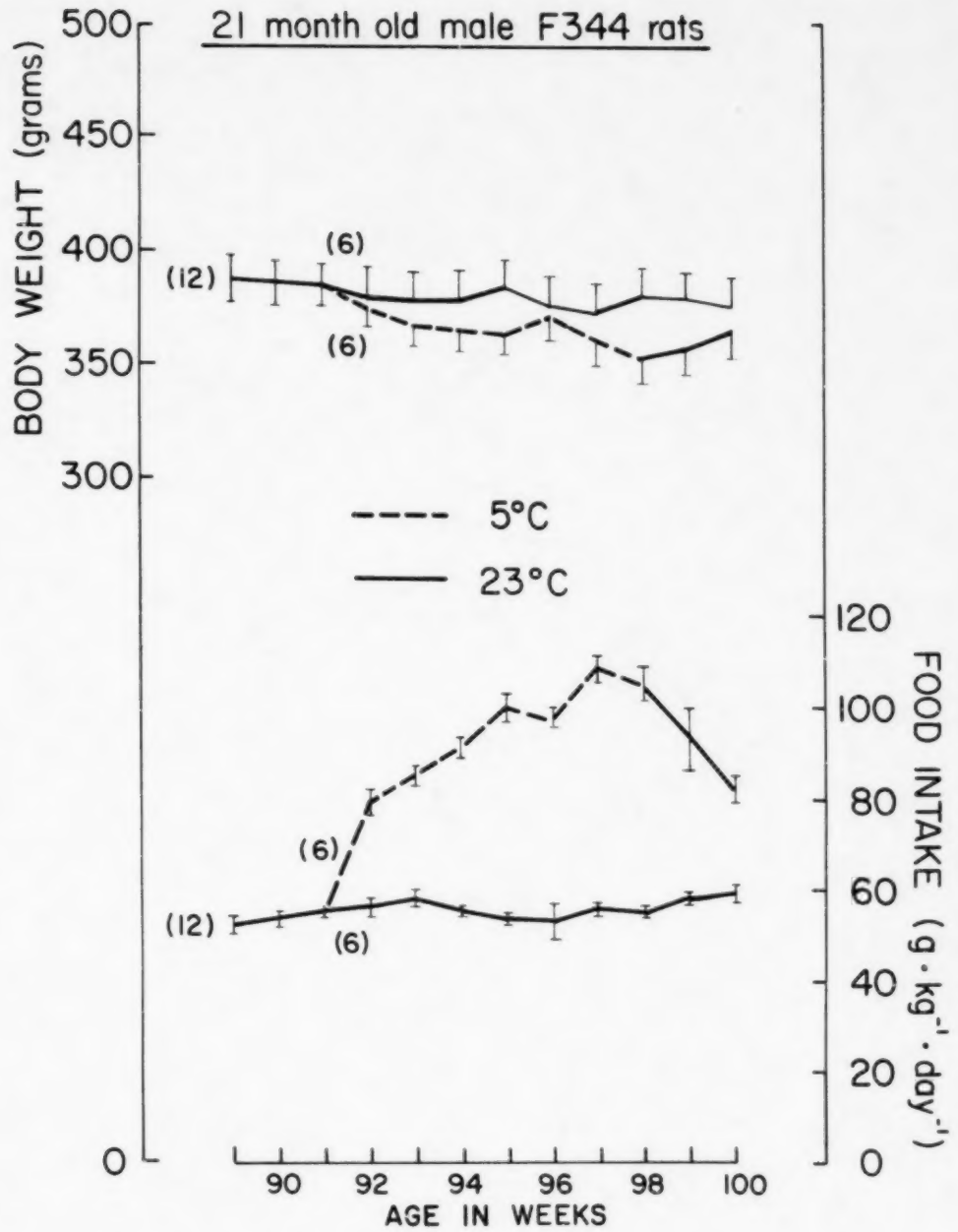

FIG. 3. Body weights and food intakes of 21-month-old male F344 rats measured weekly. Each data point represents mean ± SE. Number of animals in parentheses. The 7-week period of 5°C exposure began at week 91 and ended at week 98. These animals were then returned to 23°C.

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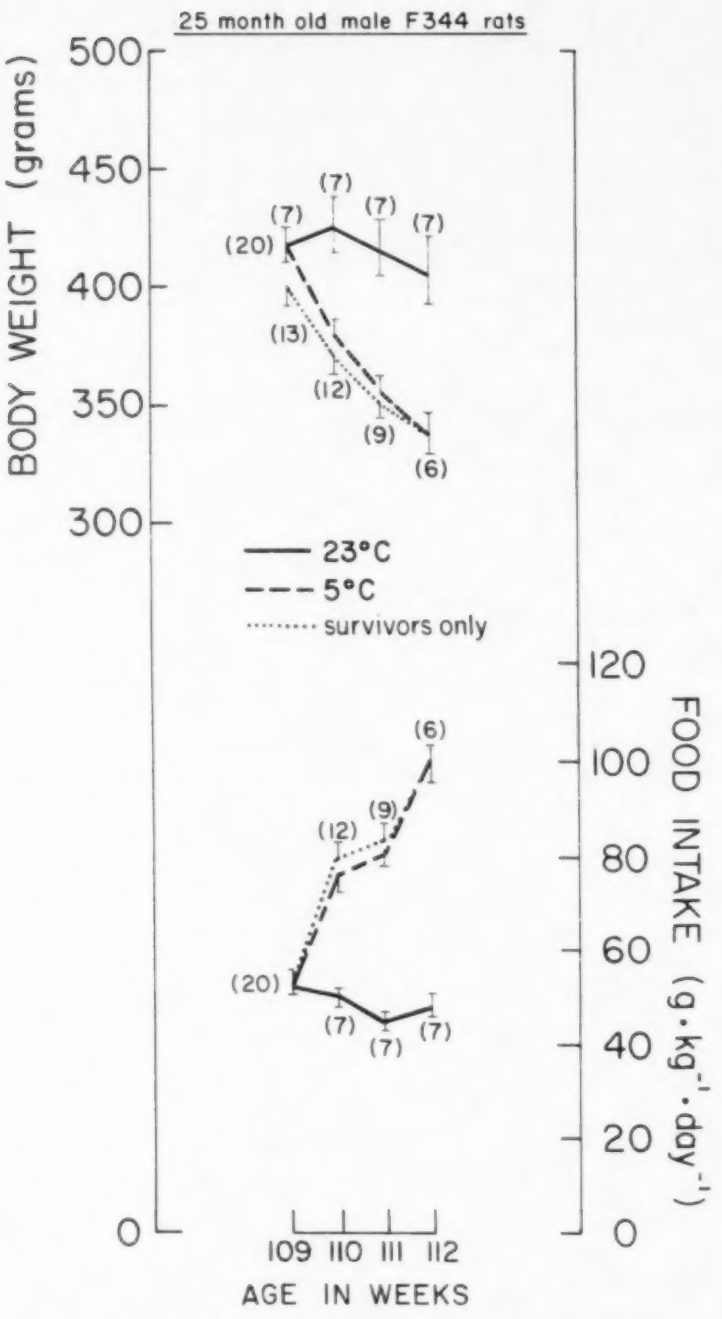

FIG. 4. Body weights and food intakes of 25-month-old male F344 rats measured weekly. Each data point represents mean ± SE. Number of animals in parentheses. The 3-week period of 5°C exposure began at week 109 and ended at week 112. These animals were then returned to 23°C.

Food intake was stable throughout the test period in non-CA rats. On exposure to 5°C, food intake increased immediately, reaching a peak value (100% increase) by 6 weeks. Both the percentage increase and the absolute amount of food intake were greater in 21-month-old rats than in the 12-month-old group in the same ambient condition (Figure 3).

25-month-old rats

Survival in the 5°C environment was reduced, as one animal each died on the 6th, 9th, 11th, 13th, and 20th days and two animals on the 18th day. At the end of the 3-week period, only 6 of 13 rats remained—a 46% survival. Pathologic analysis was not performed. It was not clear whether the failure of survival in cold was due to disease associated with aging or aging per se.

Because a large number of animals failed to survive in 5°C, analysis of variance and covariance for food intake and body weight were made on the six animals who tolerated the entire 3 weeks of exposure. However, increase of food intake and loss of body weight were similar in the entire group and in the survivors (Figure 4). Non-CA rats did not have significant changes in food intake or body weight during the same period (Figure 4).

DISCUSSION

A considerable number of studies have been conducted on cold-acclimated young male Wistar and Sprague-Dawley (S-D) rats, but information on changes in body weights and food intakes during the acclimation period is limited. Even less data is available on older animals. In general, food intake increases as the environmental temperature decreases (Brobeck, 1941). Cottle and Carlson (1954) noted that when 2-month-old S-D were placed in the cold for 1 month, they increased their food intake to meet the increased caloric demands without altering their growth rate. However, when 3-month-old S-D were placed in the cold, they did not increase their food intake to maintain growth until after 1 week in cold. With continued exposure, growth was maintained but food intake decreased in the pattern observed in 3-month-old S-D maintained at 23°C (Cottle and Carlson, 1954). We observed a similar pattern in our 3-month-old S-D (Kiang-Ulrich and Horvath, 1979). We also studied old (25-month-old) S-D, who increased their food intake appreciably in the first week in 5°C and continued to increase their food intake during the entire exposure period. However, the increase was insufficient, resulting in a steady decline of body weight (Kiang-Ulrich and Horvath, 1979).

Responses to cold in strains of rats other than S-D have received minimal attention. Recently F344 have become available for experimental uses. It is known that there are major differences in the growth pattern of F344 and S-D. F344 rats grow continuously throughout their life span to a maximum mean weight of 346 g (Chesky and Rockstein, 1976) or to 569 g (Masoro, 1980), depending on the environment and available food. S-D rats attain a maximum weight at 24 months of age, which could be from 750 g (Lesser *et al.* 1973) or to 880 g (Nolan, 1972).

We were able to compare the responses to 5°C of 3-month-old male F344 with that of a group of 3-month-old male S-D. The 3-month-old S-D grew very rapidly in 6 weeks at 23°C, and their weight increased from 212 to 431 g. When exposed to 5°C, S-D still grew, but at a slower rate than the 23°C rats (from 212 to 370 g in 6 weeks) (Figure 5). During this period the S-D in 23°C showed a linear decrease in food intake with time, resulting in

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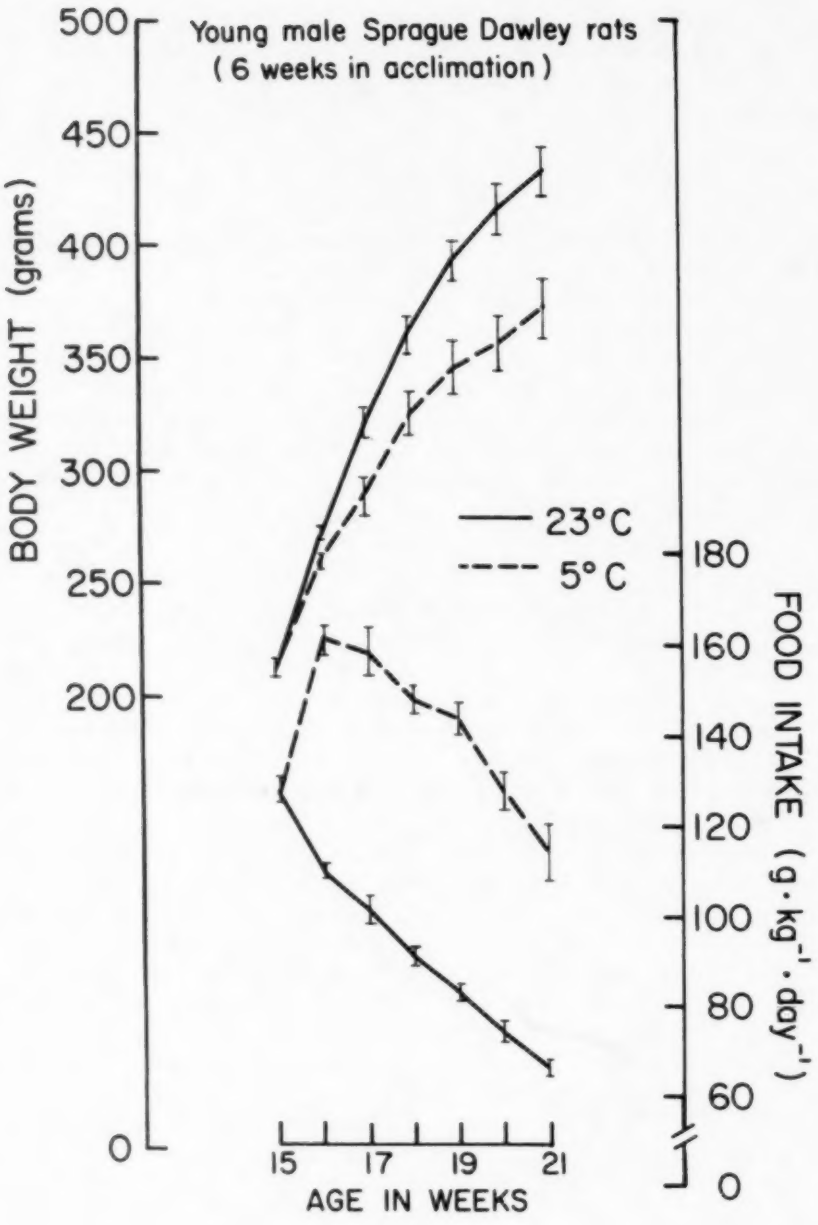

FIG. 5. Body weights and food intakes of 3-month-old male Sprague-Dawley rats measured weekly. Each data point represents mean ± SE. The 6-week period of 5°C exposure began at week 15 and ended at week 21. N = 12 in each group.

a dramatic change from 128 to 66 g • kg^{-1} • day^{-1}. S-D during their first week in 5°C had a striking increase in food intake (from 128 to 160 g • kg^{-1} • day^{-1}). However, after the first week, food intake decreased linearly with time over the next 5 weeks in a pattern similar to that observed in the 23°C rats. The food intake of 5°C S-D measured at the end of 6 weeks (114 g • kg^{-1} • day^{-1}) was still significantly higher than that of 23°C rats (66 g • kg^{-1} • day^{-1}) measured at the same time period, but was significantly less than the food intake measured 6 weeks ago before cold acclimation (Figure 5). At 3 months of age F344 also grew significantly from 272 to 318 g (vs. 212 to 431 g in S-D), though considerably less than the S-D. As F344 grew, their food intake decreased, but the decrease was not statistically significant. When F344 were exposed to 5°C their food intake increased immediately and dramatically (100% increase at peak), and the increase was maintained during the exposure. However, this increase of food intake was insufficient to support further growth, but adequate to maintain body weight (Figure 1). Thus, the energy economy of food intake and growth was greatly different not only between the 5°C and 23°C acclimated rats but also between the S-D and F344 rats at the 3-month age stage.

No information on food intake and body weight were available on 12- and 21-month-old S-D rats for comparison with 12- and 21-month-old F344 rats.

Compared to 3-month-olds, the 12-month-olds weighed significantly more but ate significantly less than the 3-month-olds, and growth appeared to be stabilized. When 12-month-olds were exposed to cold, the increased food intake was significantly less than the increase seen in 3-month-olds in a similar environment, yet body weight was maintained. Thus, 12-month-olds appeared to be more economical in food energy than the 3-month-olds.

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The increase of food intake in cold was less in the 12-month-olds than in the 21-month-olds. Furthermore, the 21-month-olds, with a higher increase in food intake than the 12-month-olds, were not able to maintain body weight as well as the 12-month-olds. They lost body weight in cold but survived.

The inability to maintain body weight in cold became very obvious in the 25-month-old group, even though their food intake was increased (Figure 4). It was at this age that survival in cold became a real issue. This age group was observed for only 3 weeks in 5°C. Only 6 out of 13 animals remained alive at the end of 3 weeks. This is in striking contrast to the younger animals, all of whom survived much more extended periods of cold exposure. It is of some interest that all the 25-month-old animals (S-D and F344) that we have studied showed the same inability to survive the cold exposure (Kiang-Ulrich and Horvath, 1979; and this paper).

Because acclimation and survival at 5°C is dependent on maintenance of an elevated caloric expenditure, an increased consumption and adequate utilization of the food provided are required. The Lab Chow we provided for the rats has a carbohydrate content of 51%. The effectiveness of glucose as a fuel of thermogenesis is demonstrated by the elevated rates of its oxidation and turnover in rats acclimated to cold (Depocas and Masironi, 1960). An adequate utilization of glucose would be prerequisite for sustained cold survival. Our 25-month-old rats were able to increase their food intake, but it is possible that they were not able to utilize the glucose as efficiently as the young rats. It is known that the glucose-stimulated secretion of insulin is progressively impaired in time of onset, magnitude, and kinetics of response (Adelman *et al.* 1978).

We are inclined to speculate that the age-related difference in energy economy and re-

sponse to cold we observed could be due to different responsiveness in brown adipose tissue (BAT) at different age stages of life and under different ambient temperature conditions. BAT has been identified as the principal site of non-shivering thermogenesis (Foster and Frydman, 1978; 1979) and exerts an important role in preserving energy balance (Rothwell and Stock, 1981).

We were surprised to note that the only other study in which body weight, food intake, and survival in 5°C were reported had completely different results from ours. Gambert and Barboriak (1982) exposed male F344 at ages 6 weeks, 6 months, and 18 months to 4–5°C for 12 days. They reported that by day 12 only 14% of 6-week-old, 50% of 6-month-old, and 71% of 18-month-old F344 survived. This is in complete contrast to our observations; we had 100% survival in 3-, 12-, and 21-month-old male F344 exposed to 5°C for 7–9 weeks. Gambert and Barboriak's 6-month-old animals increased their food intake in cold (quantity not reported) but lost 4 g • day^{-1} body weight, while their 18-month-olds decreased their food intake in cold and lost 8.9 g • day^{-1} body weight. None of our 3-, 12-, or 21-month-old rats had a decreased food intake in cold; in fact, all our rats had 80–100% increases. Gambert and Barboriak observed decreases in body weight of 6-month-olds, more so in 18-month-olds, whereas we observed no change in body weight in 3- and 12-month-olds and a slight though significant loss of weight in the 21-month-olds. They implied that the increased body weight that occurred with age might be advantageous in improving survival time, despite the fact that their cold-exposed 18-month-olds lost weight at a faster rate than the 6-month-olds due to the decreased food intake, yet survived better than the 6-month-olds. We calculated that their 6-month-olds weighed [361 − (4 × 12) = 313 g] the same as 18-month-olds [420 − (8.9 × 12) = 313 g] at the 12th day. They also speculated that the increased body fat in the older rats reduced the cold stimulus. It is known that male F344 rats are relatively lean to 20 months of age (Masaro, 1980). Gambert and Barboriak's statement "rats at no point were starving in absolute terms" (Gambert and Barboriak, 1982, p. 686) suggested they did have some doubt as to whether their rats' eating behaviors were normal. However, their finding of an increased serum T_3 in cold-exposed 6- and 18-month-olds associated with decreased conversion of T_4 to T_3 by liver homogenate with no change in serum TSH level may suggest the presence of an abnormal thyroid function in their rats. One possible source of increased serum T_3, with decreased conversion of T_4 to T_3 by liver homogenate, could be increased to T_3 from the thyroid gland.

However, we cannot present any obvious explanation for the differences reported by us and Gambert and Barboriak. We point out that all our 3-month-old S-D also survived 6 weeks 5°C exposure. Evaluation by other investigators appears to be warranted.

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CAN MUTAGENESIS REVEAL MAJOR GENES AFFECTING SENESCENCE?

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Abstract—The *Drosophila melanogaster* genome was screened for sex-linked recessive and autosomal dominant mutations affecting the senescent decline in motor abilities. No persistent identifiable genes delaying the onset of senescence were recovered among 18,089 F_1 male offspring of males exposed to radiation or the chemical mutagen, EMS.

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INTRODUCTION

A WIDELY held theory of aging is that organisms, as a consequence of natural selection, tend to accumulate genes that promote high fitness at pre-reproductive and reproductive ages but have harmful effects in post-reproductive individuals (Williams, 1957). Mutation of such genes to hypofunctional or null alleles might well promote longevity and be detected by youthful behavior in old individuals. Recovery of such mutants, however, could be hindered by early adverse effects such as decreased survival, competitive ability, mating ability, or fertility. We report, here, on the outcome of one mode of attack on this problem.

MATERIALS AND METHODS

Genes of the class postulated above could be revealed only if they are dispensable when mutated when selection is relaxed. Accordingly, offspring of mutagenized males were reared on uniformly fresh food at 25°C in the dark under uncrowded conditions. After eclosion, adult males were maintained at 20°C or 28°C; 50% relative humidity and under constant light to promote activity. Standard *Drosophila* medium (Lewis, 1960) was used at all times and adults were stored 20/shell vial, transferred, and counted every 3 days. At 25°C in constant light, adult males of the Ore-R-C strain have a mean lifespan of 56.3 days (maximum 74 days) while at 28°C the mean is 35.7 days (maximum 46 days). At both temperatures, survival curves approached the classic rectangular shape and were reproducible from generation to generation.

An attached-X chromosome bearing the recessive markers *y* (yellow body) and *f* (forked bristles) had been placed in the Ore-R-C background by 10 generations of backcrossing (genetic variants are described in Lindsley and Grell, 1968). Females of this strain were crossed to mutagenized Ore-R-C males, and cohorts of F_1 males were collected daily and maintained at 25°C or 28°C. The latter temperature considerably shortens the lifespan and simplifies the logistics of this experiment but is at the borderline of physiological tolerance (Hollingsworth, 1969); accordingly, we used an acclimated substrain of Ore-R-C reared at 28°C for 10 generations. Male parents of the male flies to be aged and tested for new mutations were either fed the alkylating agent EMS (ethyl methane sulfonate in alternating doses of 0.006 *M* and .0125 *M* according to the procedure of Lewis and Backer,

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TABLE 1.

Mutagen	Adult temperature centigrade	Number of males screened	F_1 Unusual climbers recovered	F_1 Mated and fertile	Lines of unusual climbers F_2	F_3	F_4	F_5	F_6
EMS	25°	5,511	9	3	1	1	1	1	0
	28°	6,775	25	4	2	1	0	–	–
X-rays	28°	5,803	11	4	1	0	–	–	–
TOTAL		18,089							

1968) or X-rayed at 1800 R (delivered within 5 minutes). Exposure to the mutagens was adjusted by checking that the sex ratio was approximately 1:1 and that the mean lifespan of experimental animals was not significantly different from that of controls at the appropriate temperature. Use of the attached-X chromosome permitted testing for sex-linked recessive genes affecting behavior in only one generation. Autosomal dominant genes (but not autosomal recessives) would also be detectable.

At a time approximately three-quarters through the adult life span, the 60-200 males of an aged cohort consisting of 1 day's collection were tested for climbing ability by placing them without anesthesia in a 500 cc cylinder, and tapping the cylinder base sharply three times against the table. Those few climbing to the 250 cc level within 20 seconds were removed (by suction), retested, and if unusual climbing ability was confirmed, individually housed in a vial for 4 hours with several receptive virgins of the same attached-X strain. In the F_2 and later generations, the sexes were reared separately and tested for climbing ability in order to determine whether a sex-linked or autosomal pattern of inheritance was present.

RESULTS

Decline in (negative geotactic) climbing abilities and increases in time required to mate (mating latency) were pronounced at three-quarters of the mean adult life span as reported by Herman *et al.* (1971). Most of the 18,089 flies screened failed to pass the double test, and of those older males that climbed with a youthful speed, almost three-quarters had a mating latency greater than 4 hours (or were sterile).

No fertile F_1 male crossed to attached-X females displayed an X-linked pattern of climbing ability in his F_2 progeny. Of the four F_2 lines that did continue to display unusual negative geotactic response, only two did so in the F_3. One was a female recovered at 28°C that failed to pass on her climbing ability to the F_4. The other was a 25°C line that showed marginal climbing ability in the F_2 but strong negative geotaxis in half the aged F_3 of both sexes, suggestive of an autosomal dominant gene. However, the proportion of climbers diminished progressively in the F_4 and F_5. This line was discarded when no unusual motor patterns were detected after testing the aged F_6 (Table 1).

DISCUSSION

Identification of a major gene (or genes) capable of delaying senescence and extending lifespan could greatly facilitate inquiry into causes of senescence. Such genes have not yet been revealed by artificial selection for longevity (Comfort, 1953; Wattiaux, 1968; Lints *et al.*, 1979). It is possible that genetic variability for this class of gene is low or absent in most populations. Mutagens, however, might generate anti-senescence alleles and the proper selection procedure reveal them.

One possible interpretation of the results of this experiment is that there are no major genes capable of mutating to alleles that can be recovered as promotors of youthful motor

behavior in old flies. Another possibility is that such genes are present but are potential recessive mutations in the four-fifths of the genome, the autosomes, that remain unscreened in the present study. A third possibiiity is that mutants of the desired type were produced but were not detected because of their linkage with simultaneously induced subvital or detrimental mutations. To reduce this effect, we adjusted mutagen exposures down until survival curves of experimental and control animals were comparable, but we cannot completely exclude this possibility. It is conceivable that a larger scale experiment or one that allows subvitals to segregate from late acting beneficial alleles might reveal key loci responsible for senescent decline of motor abilities.

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THE CONSEQUENCES OF FAT BODY TRANSPLANTATION INTO YOUNG AND OLD *DROSOPHILA*

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Abstract—Despite some age-related changes in cells, we observed no massive fat body involution during senescence of two species of *Drosophila*. Transplants of larval fat failed to prolong the life of old *D. gibberosa*. Transplanted larval fat persisted for weeks and underwent similar changes (except for glycogen stores) in old as in young hosts, suggesting that only minor changes in hemolymph composition occur with age.

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INTRODUCTION

FAILURE OF a vital organ system such as the insect fat body, the major organ of intermediary metabolism (an analogue of the mammalian liver), could, if progressive and late in life, account for senescence and death. A report that the fat body in aged *Drosophila melanogaster* is usually atrophic and may, therefore, play an essential role in senescence (Miquel, 1971) led to our examination of the structure of intact and transplanted fat bodies during aging of two species of *Drosophila*.

MATERIALS AND METHODS

Drosophila melanogaster (Oregon R-C) were cultured under uncrowded conditions (20 or fewer per vial) in constant light (to maximize activity) at 25°C (mean lifespan, 62.2 days) or at 28°C (mean lifespan, 33.1 days). *Drosophila gibberosa* were cultured at 25°C (mean lifespan 81.2 days) in the dark except for periodic exposure to light (for mating or for changing to new vials and food) every 3 days. Standard *Drosophila* medium was used for both species (Lewis, 1960). Under these conditions, mortality curves approach the classic rectangular shape. *D. gibberosa* (like *D. melanogaster*) exhibits the usual signs of senescence after reaching mean lifespan: decreased life expectancy, high sensitivity to trauma, deteriorated motor abilities (climbing, mating, righting if overturned) as well as the cellular changes described in this paper.

Fat for transplantation was taken from either 5-day-old adult *D. gibberosa* (one experiment) or from 5 to 6-day-old larvae (most experiments). Dissections were made in sterile Shen's solution (9 gm NaCl, 0.42 gm KCl, 0.25 gm $CaCl_2$ in 1 L H_2O) and within 30 minutes, fat was injected into adult thorax or abdomen using sterilized glass capillaries drawn and ground to fine, beveled points. Fat was injected into young (10 to 24-day) or old (90 to 135-day) adult *D. gibberosa*.

In one series of experiments designed to test the effects of fat body transplants on host longevity, plates of 30-60 large cells from posterior lobes of the larval fat body (but not including gonads) from a 5 to 6 day-old (mid-third instar) larva were implanted into the thorax or abdomen of old *D. gibberosa*. Longevity of recipients of fat was compared with that of sibling-controls that had received only sterile Shen's solution (or were uninjected controls).

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Another series of experiments were designed to test the effects of the internal environment of young and old hosts on the transplant. Fat body plates of 30-60 large cells (without gonads) from a single 5 to 6-day-old larva were transplanted, alternately, into young or old hosts. A control fat body lobe was fixed, for study, at the end of the injection period. At intervals from 10 days to 3 weeks (usually 14 days), hosts were dissected in Shen's solution; the transplanted fat removed and processed immediately for electron microscopic examination. Experimental and control animals were marked by removing scutellar bristles and housed in the same vial (at 25°C, in the dark to decrease activity) to provide uniform culture conditions.

For light microscopy, young (age 7 days) or senescent (age 30 days after culture at 28°C, under constant light, or age 60 days after culture at 25°C under constant light), adult *D. melanogaster* were lightly anesthetized with ether. The head was severed from the thorax and abdomen and the proboscis removed. A hole was made in the thorax; and the head, thorax, and abdomen fixed in a formaldehyde-glutaraldehyde fixative for 2 hours (Karnovsky, 1965). Paraffin-embedded sections were stained with Haematoxylin-Eosin.

For electron microscopy, tissues were dissected in Shen's solution and quickly fixed in 5% glutaraldehyde with 0.1 *M* cacodylate buffer (pH 7.4) for 4 hours. The tissue was then rinsed twice in 0.1 *M* cacodylate buffer, postfixed overnight in buffered OsO_4, washed, and progressively dehydrated in graded alcohols. Tissues were embedded in Spurr's medium (Spurr, 1969), sectioned with a Porter-Blum M.T. 2B, mounted on copper grids, stained with lead citrate (with or without uranyl acetate), and examined with a Phillips 200 electron microscope.

RESULTS

Fat body cells from young adult males of *D. gibberosa* (Figure 1) are replete with glycogen granules and lipid droplets, which, in fact, account for most of the cell volume. The degree of cell-to-cell variation is somewhat greater in fat bodies of old flies. Adjacent cells, however, may differ markedly in the density of stored glycogen and lipid in both

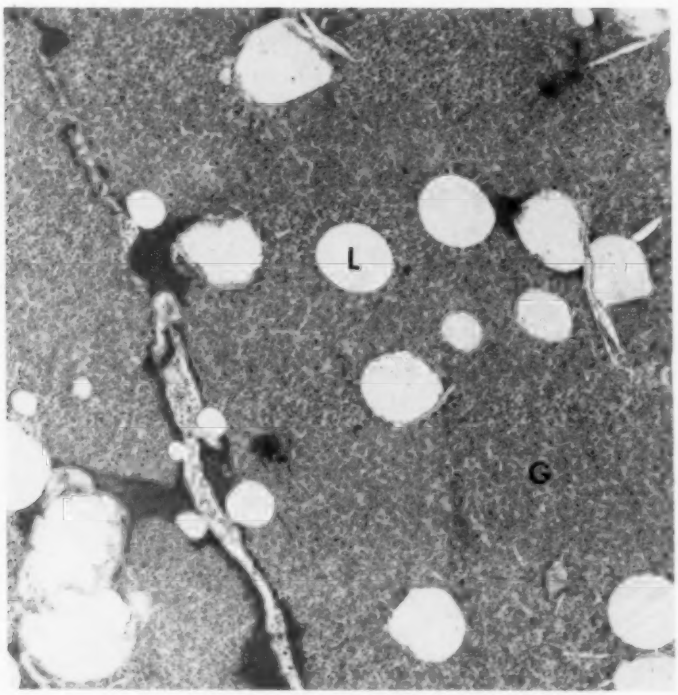

FIG. 1. Electron micrograph of part of two cells of abdominal fat body of young male. Cell boundary stretches from upper left to lower center. Most of cell is packed with glycogen (G) and lipid (L). (× 2900)

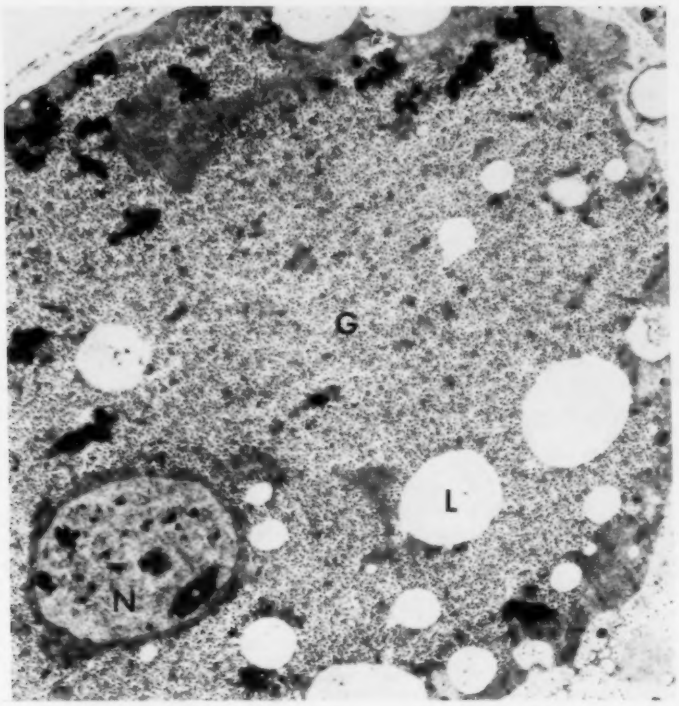

FIG. 2. Electron micrograph of abdominal fat cells of old male. Addition of electron-opaque material represents major change from the young fat body of Fig. 1. Note small size of nucleus (N) in adult fat. Adjacent cell (bottom right) has contents of much lower density. (× 2150)

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young and old flies. This may reflect cell-to-cell differences in metabolism or viability (see Discussion). Dense, osmiophilic bodies (Figure 2) appear in the cytoplasm as noted by Miquel (1971) in *D. melanogaster*, and the mitochondria appear more abnormal with less distinct cristae in old flies. Glycogen and lipid are by no means exhausted in old flies, nor are fat bodies shrunken and atrophic. The oldest (146-day) flies that we dissected (barely able to walk, not able to fly, but feeding regularly) still had significant amounts of fat body remaining.

Serial sections of heads and abdomens ($N = 2$ for young, $N = 6$ for aged) from young (4 to 6-day-old) *D. melanogaster* when compared with those of senescent flies (one month at 28°C in constant light, mean life expectancy 33.1 days; 2 months at 25°C, constant light, mean life expectancy 62.2 days) reveal no depletion or involution of fat bodies in any way comparable to that described by Miquel (1971) in 70 to 104 day-old flies reared at 22°C.

Transplants of fat removed from early to mid-third instar larvae of *D. gibberosa* are replete with glycogen, lipid, and dense protein bodies (Figure 3). Transplants persist, if not indefinitely, up to a month in old flies (see following) but do not prolong the life of old *D. gibberosa* (Table 1). Old individuals show high mortality when injected with either fat in Shen's solution or Shen's solution alone (compare injected with uninjected controls.) Sen-

TABLE 1.

Material injected	*Number of aged flies (90 to 135-days-old) treated*	*Mean survival, in days after injection*
Larval fat	47	9.9
Shen's solution	48	10.3
Uninjected control	9	23.9

sitivity of old flies to injection trauma (and, perhaps infection, despite sterile precautions) is much greater than that of young flies.

Adult fat transplanted into adults can be recovered after 1–2 weeks but appears quite degenerate. We attribute this to severance of the usually heavy tracheation of adult fat when it is transplanted.

Larval fat transplanted into young and old hosts can persist at least as long as 26 days. There is no tendency for transplanted fat to be lost prematurely from old as compared with young hosts. Transplants, however, were usually removed from hosts approximately 2 weeks after implantation. Comparison of transplant ultrastructure with control fat

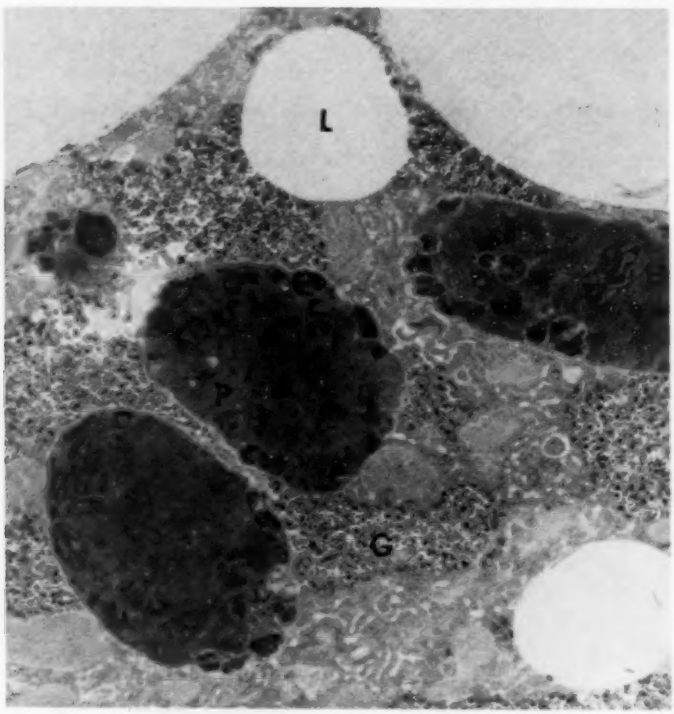

FIG. 3. Electron micrograph of control fat body from 5 to 6-day-old larva before transplantation into adult. In addition to glycogen (G) and lipid (L) larval fat cells have dense protein bodies (P). (× 4750)

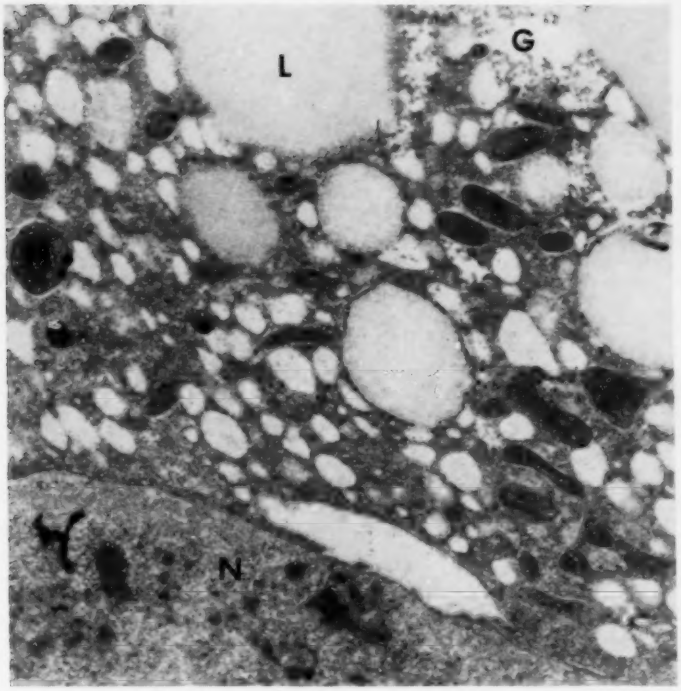

FIG. 4. Electron micrograph of larval fat body transplant recovered after 16 days in hemolymph of a young (14–30)-day adult male fly. Large nucleus (N) with polytene chromosomes is at bottom. Lipid droplets (L) of various sizes, endoplasmic reticulum and mitochondria (M) make up most of volume formerly occupied by glycogen (and lipid). (× 4750)

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($N = 6$ for each age) revealed similar changes in implants into young and old hosts: complete loss of protein bodies, and replacement of a few large by many small lipid droplets. Glycogen disappears from most areas of larval fat implanted in young hosts (Figure 4) but persists at a lower density, in larval fat implanted in old hosts (Figure 5).

DISCUSSION

The accumulation of dense osmiophilic bodies (as well as subtle changes in mitochondria) with increasing age of adult *Drosophila* fat have been reported previously (Miquel, 1971). We are able to confirm these observations but as outlined above, our observations in *D. melanogaster* and *D. gibberosa* are not in agreement with Miquel's suggestion that generalized and massive atrophy of fat bodies is a regular feature of fly senescence.

The accumulation of age-specific structural alterations in adult fat body cells suggested to us the possibility of transplanting adult fat from one developmental stage to another. This should enable one to determine whether a developmental program leading, for example, to the changes illustrated in Figure 2 was intrinsic to the fat body or extrinsic and alterable. Unfortunately, we quickly found that the heavily tracheated adult fat body appeared quite degenerate following severance of the tracheae, transplantation, and

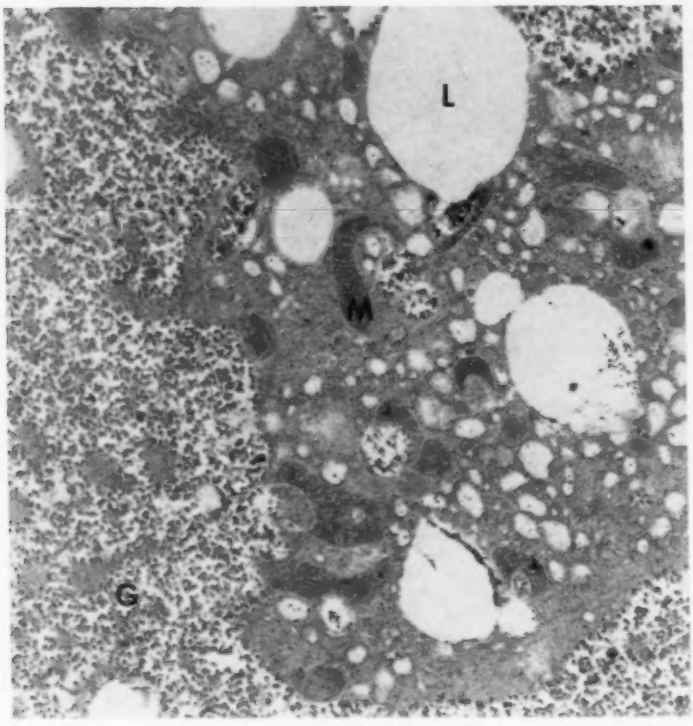

FIG. 5. Electron micrograph of larval fat body transplant recovered after 16 days in hemolymph of an old (96–112-day) adult male fly. Appearance is similar to Fig. 4 but more glycogen (G) is present. There is no electron-opaque material and cristae of mitochondria are as distinct as in Fig. 4. (× 4750)

recovery after 2 weeks. Moreover, it was difficult to distinguish transplanted adult fat from native adult fat unless transplants were made into a region such as the thorax, where fat is not abundant.

Larval fat is under a different developmental program. Plates of fat cells grow to large size in larvae but remain untracheated. Plates dissociate into single cells in early pupae; many cells of which persist through metamorphosis until adult eclosion. Larval fat cells then ordinarily lyse during the first 3 days of adult life (Butterworth and La Tendresse, 1973) under the influence of juvenile hormone (Postlethwait and Jones, 1978). However, much of the fat cell plates removed from larvae and transplanted into older adults persist despite a regular, stochastic loss of some individual cells over many days (Butterworth, 1973). Adult fat body cells arise from proliferating imaginal disc masses as larval fat declines. Adult fat nuclei are small compared with the large, polytene chromosome bearing larval fat body nuclei. Nuclei, therefore, serve as stage markers when fat transplants are retrieved (Compare Figure 2 with Figure 4).

We transplanted larval fat obtained well before metamorphosis into adults well beyond the lytic period of the young adult. Hence, 50–75% of transplants into either young or old adults could be found, and removed as plates. A few cells were removed from a plate and

checked under phase optics for polytene chromosomes; if positive, we proceeded with processing of the remainder of the tissue for electron microscopy. Most recovered transplants had fewer cells than were injected; attributable in part to damage during transplantation. But transplanted fat cells may be dying singly due to an internal program rather than *en masse* owing to the hormonal fluxes of metamorphosis (Butterworth, 1973). Consistent with this interpretation were our observations of occasional cells with depleted contents adjacent to cells of normal appearance (like that shown in Figure 2). Some cells in several transplants were larger than normal; the larger than normal size of their polytene chromosomes was consistent with extra growth in the transplant. No consistent differences in the gross nature of the transplants or their persistence were found in young versus old adults.

The presence of acid phosphatase in protein bodies and the near-simultaneous disappearance of protein bodies with the larval fat body cells in the lytic environment of the just enclosed adult led Butterworth (1973) to propose (following Gaudecker, 1963) that the protein bodies (Figure 3) are lysosomes and are responsible for fat body cell lysis. Our observations are not in agreement with this hypothesis. Although protein bodies have in all cases disappeared from larval fat body transplants (Figures 4 and 5), fat body cells without these protein bodies can persist for at least 2 weeks after protein bodies disappear. Therefore, the disappearance of protein bodies in the lytic environment of the young adult is coincidental with, but not the cause of, cell lysis.

What can we conclude from these observations about the relative contributions of extrinsic (humoral) or cell-intrinsic factors to senescence? No more electron-dense "age pigment" is found in larval fat recovered from old hosts than from young hosts; age pigment accumulation can, therefore, he attributed either to slow-acting intrinsic factors or to slow-acting extrinsic factors but not to fast-acting extrinsic factors (assuming that larval fat is susceptible). Regulation of the fate of cell storage products in transplants, however, appears to be due primarily to factors extrinsic to cells, most probably in the hemolymph.

Protein bodies disappear from all transplants. This is consistent with Butterworth and Bodenstein's (1967) report of dependence of protein deposition on larval ring gland secretion.

Dense stores of glycogen within transplanted larval fat either decline markedly (in young adults) or become less dense (in old adults). The persistence of large areas devoted to glycogen stores in larval fat taken from old hosts is the most consistent host age effect on transplants. It suggests some regulatory differences in hemolymph of old flies and may reflect lower levels of brain glycogen mobilizing factors (Goldsworthy, 1971).

Proliferation of the cytosol by endoplasmic reticulum and mitochondria (Figures 4 and 5) into areas formerly packed with glycogen, together with the appearance of numerous small vacuoles (some of which are confluent with larger lipid droplets), suggests intensified protein and lipid synthesis in transplants; however, further studies are needed to confirm this. Mitochondria appear normal in transplants from old as well as young hosts.

The homeostatic ability of old flies is markedly impaired. But larval fat transplants persist well and have similar gross morphology as well as ultrastructure, whether recovered from young or old hosts. Senescent changes are found throughout several tissues of the aged fly (Miquel, 1972). One way this might come about is through progressive alteration or deterioration of hemolymph with age. Hemolymph transplants have suggested that this occurs (Sondhi, 1966). Our observations on glycogen stores may be used to argue that aged hemolymph differs from young hemolymph, but the overall structural similarity of

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transplants recovered from young and old hosts suggests that the hemolymph of the senescent adult has not deteriorated markedly from that of the young adult.

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ACCELERATED AGING OF THE INSECT *DROSOPHILA MELANOGASTER* BY γ IRRADIATIONS OF PUPAE

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Abstract—The purpose of this study was to induce accelerated aging in the fly *Drosophila melanogaster* (Diptera) using free radicals, and to distinguish aging from radiation syndrome. After irradiation of 12 to 24-hour-old pupae with doses of 500–2500 rad, very similar fly emergence rates were observed in control and treated populations. The life span was shortened and dose-related in mixed populations and in both virgin and mated male and female populations. Males were more radiosensitive than females. In the 500–2500 rad dose range, all the populations tested were fertile after irradiation. Young treated as pupae and control adults both displayed similar mating ability and negative geotactic responses. Their response levels decreased with time, but the decline was faster and dose-related in the treated animals. This study demonstrates that irradiation of 12 to 24-hour-old pupae with doses of 500–2500 rad induces accelerated aging, without causing serious biological damage. For example, after an irradiation with 1750 rad, the "lethal time" (LT) 50 dropped by about 30%.

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INTRODUCTION

FOR SEVERAL years it has been assumed that free radicals have an important role in aging (Harman, 1982) and this subject has been investigated in different animals. As various authors have maintained, the insect *Drosophila melanogaster* (Diptera) is well adapted to this study (Lamb, 1965, 1978; Miquel *et al.*, 1972; Schnebel and Grossfield, 1983), since large populations are easily available and easy to rear in the laboratory, the adults live for several weeks and even several months, and the species is genetically well known.

The effects of ionizing radiations on life shortening have been well established in both mammals and in insects, especially *D. melanogaster* (Lamb, 1965, 1970; Nöthel, 1965; Maynard Smith, 1966; Baxter and Blair, 1967a, b; Atlan *et al.*, 1969; Miquel *et al.*, 1972; Gartner, 1973a, b; Giess and Planel 1977; Giess *et al.*, 1980). However, in insects, only high doses shorten life but they also cause serious damage known as "radiation syndrome" (Atlan *et al.*, 1969; Miquel *et al.*, 1972; Gartner, 1973b). In the present work, we attempted to distinguish between these two processes and to accelerate aging while restricting other physiological damage to a minimum. For this purpose, pupae were irradiated and the life span of emerged flies was observed. A behavioral study based on the usual criteria of

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mating ability and negative geotaxis (Herman *et al.*, 1971; Samis *et al.*, 1981) was also performed to try to separate accelerated normal processes from new processes which might be indications of irradiation damage. As noted many years ago in this connection by Bacq (1961), the process of normal aging is not yet well understood, and it is difficult to discern whether irradiation only accelerates normal vital processes, or whether it introduces new factors which shorten the life span.

MATERIALS AND METHODS

Oregon flies were raised on axenic cornflour and yeast-based medium (David, 1962), in glass bottles at a constant temperature (24 ± 1°C), with a relative humidity of 70%, and a natural photoperiod. Larvae were collected and the sexes were identified and separated for experiments involving virgin males and females, at the time of puparium formation.

The pupae irradiated were 12 to 24-hours-old, as the results obtained with older pupae proved very difficult to interpret.

Gamma irradiations were made in a ^{60}Co irradiator. Its dosimetry was determined by Fricke's method taking G = 15.6 molec/100 eV and $E_{304} = 2204\ mol^{-1} \times l \times cm^{-1}$. Doses were delivered at a dose rate equal to 500 rad min^{-1}. The doses tested were in the range of 500–3100 rad. Pupae were irradiated in pyrex tubes and were then separated into groups of five individuals and kept in glass tubes at 24°C. When the flies emerged, their sexes were checked once more, without anesthesia. The adults were reared in glass bottles (Ephrussi model) on David's medium. The effects of each dose were studied in the following three groups of flies.

1) One group of males and females in the same bottle. This condition will be called "mixed populations."
2) One group of males alone (virgin males).
3) One group of females alone (virgin females).

Three control groups, one for each experimental condition, were simultaneously studied.

There were about 120 flies per bottle. Mortality was noted daily. Insects were transferred on fresh medium every 2 days, until the last fly died. Each experiment was repeated from 2 to 4 times and about 5500 pupae were used altogether.

For behavioral investigations such as the response to negative geotaxis and mating ability, males (control or irradiated as pupae) were also reared in glass tubes containing David's medium (10 flies per tube). The medium was changed every 4 days.

Negative geotaxis

These were performed once a week in a 250 ml graduated cylinder with a diameter of about 3 cm. Ten males were used for each measure. To force them down to the bottom of the cylinder, the latter was struck three times on a hard rubber cushion. The number of flies which rose higher than the 150 ml mark within 20 seconds of the last strike was counted. This procedure was repeated five times with the same flies and the mean of the number rising beyond the 150 ml mark was used for percentage calculation. The initial number of flies was about 800.

Mating ability measures

Ten virgin males were placed in an empty glass tube with 15 virgin females 4-days-old, since at that age, the females are highly attractive (Antony and Jallon, 1982). The number of copulating males was recorded for the 10 minutes after the introduction of the females. As copulation lasts more than 10 minutes in this species, there was no risk that any male would be counted twice. In all, about 1150 control males and about 500 treated males were investigated. Tests were conducted once a week, and as only virgin males were used, the males were discarded after each test.

Both geotaxis and mating ability were always measured in the middle of the afternoon, to avoid rythmic behavioral response (Samis *et al.*, 1981).

On the survival curves, the "lethal time 50" (LT 50) which indicates the number of days required for 50% of the flies to die, was examined with particular attention. For statistical analysis of the results, analysis of variance and Student's *t* test were used.

RESULTS

The emergence rates of the flies irradiated as pupae are given in Figure 1. The curve shows that, up to dose of 2100 rad, the rates of emergence remained normal, since they were similar in control and treated flies; for low doses, emergence rates were even higher

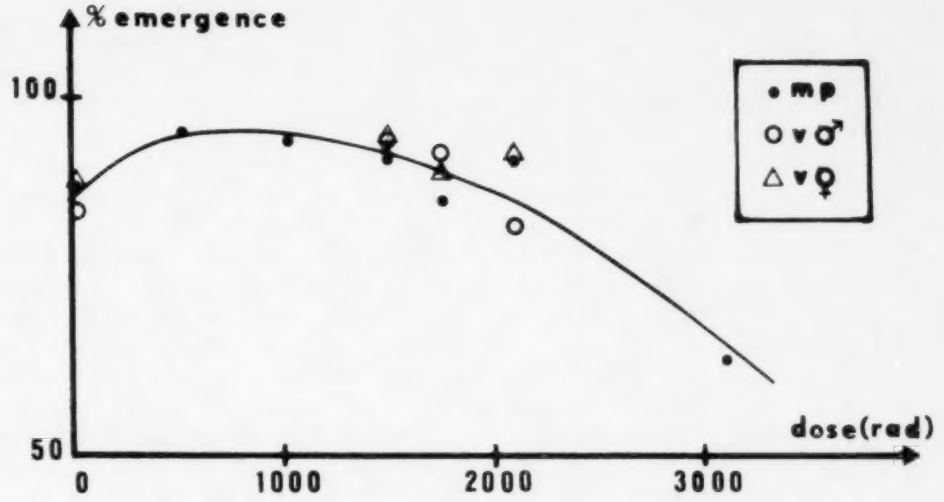

FIG. 1. Emergence rate of flies in relation to the dose of irradiation during the pupal stage; mp = mixed population; v ♂ or ♀ = virgin males or females (each point represents about 325 pupae).

than normal in the treated group. However, when the dose reached 3100 rad, the emergence rate in mixed populations feli by 37%.

For mixed populations, four survival curves from the many obtained are shown in Figure 2. They indicate that the three treated populations had a reduced life span. Thus, a statistically significant diminution of the LT 50 value was observed (530 rad : 33.93 ± 1 day; 2100 rad : 22.6 ± 0.8 d; 3100 rad : 16.48 ± 1.5 d; controls : 37.05 ± 0.58 d, that is, a ratio of treated/control LT 50 reduction in the range of 8.4% to 54.7%). The higher is the dose, the smaller is the LT 50 value. Three of the curves show the commonly observed rectangular shape while the fourth (3100 rad) is more linear.

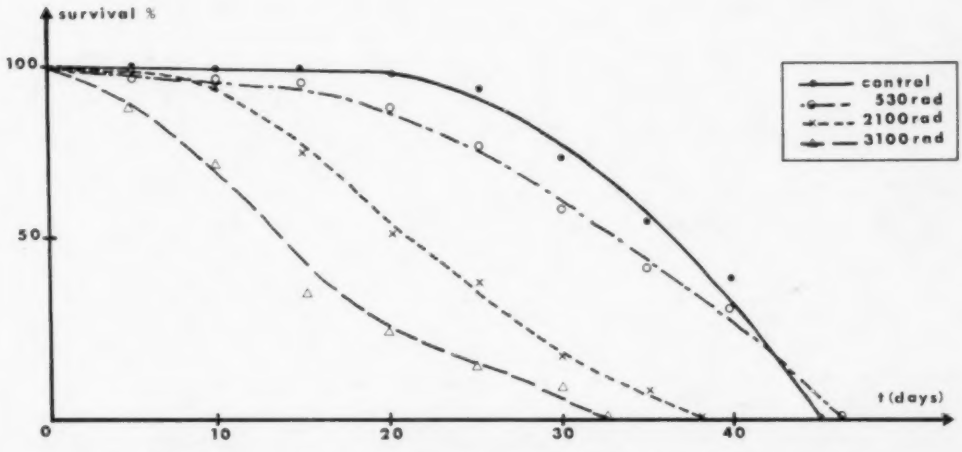

FIG. 2. Survival curves of flies emerged from control and irradiated pupae: mixed populations.

The dose effect observed in Figure 2 was noted in all the repeat experiments. The regression line in Figure 3 shows that, in mixed populations, the LT 50 values correlated with the dose. All the populations treated with doses up to 2500 rad produced offspring. On the other hand, the populations irradiated with 3100 rad were completely sterile. At this dose, the sterility observed, as well as the high level of nonemerging individuals, the 60% drop in LT 50 and the almost linear survival curve (Figure 2), all suggested that important physiological changes occurred in the treated animals. Consequently this dose was too high for the purposes of this study and was not used in subsequent experiments.

The radiosensitivity of virgin males and females was tested with various doses, three of which were finally selected for irradiation of the pupae (1500, 1750 and 2100 rad). Figure 4a-b gives the results for the 1750 rad dose. On this figure, the survival periods for virgin control or treated flies are compared with those for mated control or treated flies (linear regression after probit-log transformation of the data). Because of the respective slopes of the curves (Figure 4a), they form pairs, both for the virgin control or treated males and for the mated control or treated males; the survival curves are therefore similar for each of these two groups. However, in each one, the distance between the two curves, and the statistically significant differences in LT 50 values, show a clear reduction in the survival of the irradiated populations. Figure 4a also shows that mated males are more radiosensitive than virgin males and that mated control males have a shorter life expectancy than virgin control males. The same conclusions also apply to the females, because as shown in Figure 4b, both control and irradiated mated females have a shorter life expectancy than virgin females, whether control or irradiated.

A radiation dose effect on the LT 50 was noted for virgin males and females, as well as

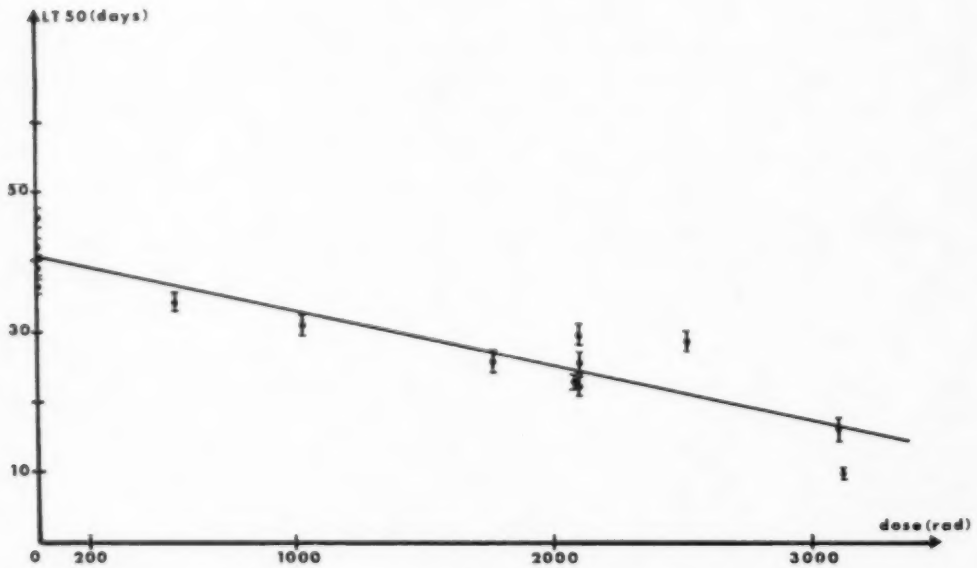

FIG. 3. Dose effect in mixed populations : regression line of the LT 50 values plotted against the dose of irradiation; $y = -0.008 \chi + 41$; $r = 0.921$ (12 df); $P < 0.01$.

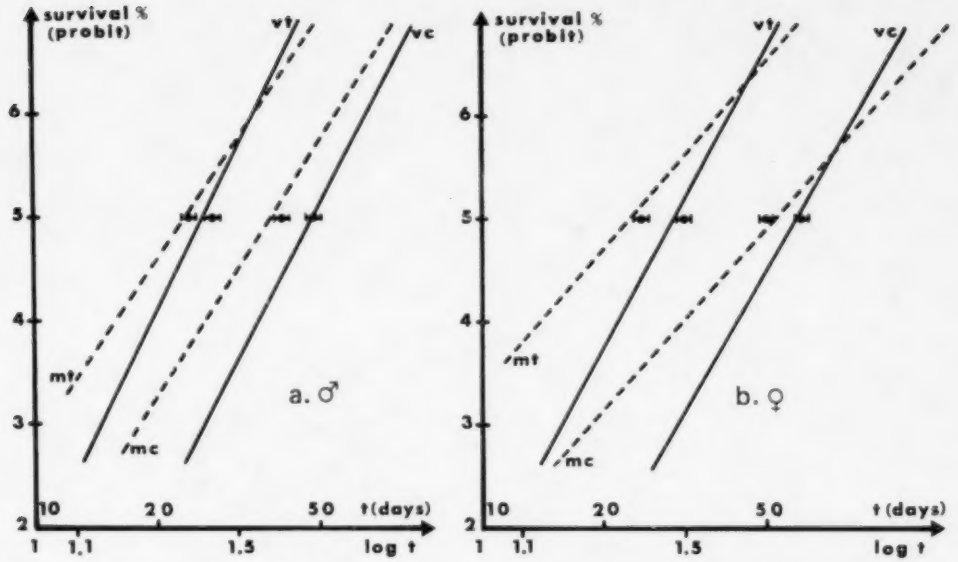

FIG. 4. Survival of flies emerged from pupae irradiated with 1750 rad. Percentages of survival are plotted against time (Probit-log transformation of the data); confidence intervals of the calculated LT 50 values are indicated.

a) vc ♂ = virgin control males; $y = 7.5\ t - 7.65$; $r = 0.829$ (8 df); $P < 0.01$.
vt ♂ = virgin treated males; $y = 7.83\ t - 6.13$; $r = 0.994$ (6 df); $P < 0.01$.
mc ♂ = mated control males; $y = 6.24\ t - 4.86$; $r = 0.951$ (9 df); $P < 0.01$.
mt ♂ = mated treated males; $y = 5.81\ t - 2.95$; $r = 0.99$ (7 df); $P < 0.01$.

b) vc ♀ = virgin control females; $y = 6.61\ t - 6.78$; $r = 0.822$ (9 df); $P < 0.01$.
vt ♀ = virgin treated females; $y = 7.39\ t - 5.83$; $r = 0.96$ (9 df); $P < 0.01$.
mc ♀ = mated control females; $y = 4.39\ t - 2.53$; $r = 0.884$ (9 df); $P < 0.01$.
mt ♀ = mated treated females; $y = 4.44\ t - 1.06$; $r = 0.99$ (8 df); $P < 0.01$.

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mated males and females. Figure 5 shows the four corresponding regression lines. The males were more radiosensitive than the females, but the difference decreased at high doses.

As a rule, only males are used to study geotaxis and mating ability. Females, even when virgin, cannot be tested, as they always undergo vitellogenesis, which increases their body weight.

For the present studies, virgin males were kept 10 to a vial. Although this reduced density increased the longevity of both treated and control males, the ratio of treated to control LT 50 reduction was the same as that reported previously for treated and control populations of higher density, that is, 11% to 45% according to the dose.

The usual tests for aging, geotactic responses and mating ability, were applied to the virgin males irradiated as pupae. Figure 6 shows the results observed. With a dose of 2100 rad, the negative geotactic response of the males began to decrease as soon as 1 week after emergence. This trend intensified and became statistically significant during the following week. With a dose of 1750 rad, reduced negative geotaxis was only observed 2 weeks after emergence, and the reduction became statistically significant at 3 weeks. With a dose of 1500 rad, the reduction did not appear until 3 weeks after emergence, reaching statistical significance after 4 weeks. Thus, a dose effect was also demonstrated for geotaxis.

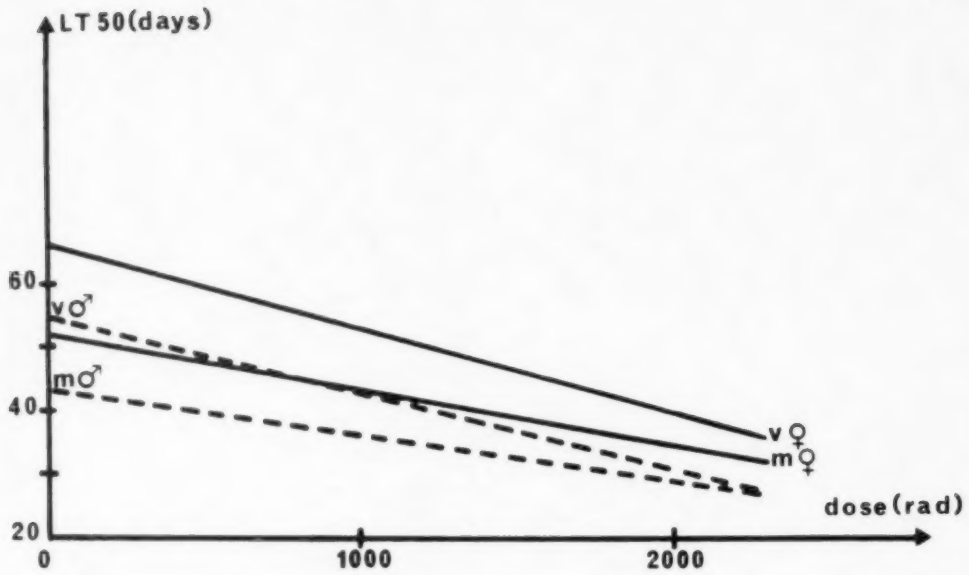

FIG. 5. Dose effect in virgin and mated flies. Regression lines of the LT 50 values are plotted against the dose of irradiation.
v ♂ = virgin males; $y = -0.012x + 54.63$; $r = 0.864$ (7 df); $P < 0.01$.
m ♂ = mated males; $y = -0.008x + 43.74$; $r = 0.967$ (7 df); $P < 0.01$.
v ♀ = virgin females; $y = -0.014x + 67.6$; $r = 0.771$ (4 df); $0.05 < P < 0.1$
m ♀ = mated females; $y = -0.009x + 51.78$; $r = 0.726$ (4 df); $P \cong 0.1$.

The mating ability curves are more complex. The fluctuations were fairly large during the 4 weeks following emergence. After this period, a dose effect can be seen. The mating ability of males irradiated as pupae with a dose of 2100 rad is not reported on the graph, because only 10 to 15 of these males were tested each week. For 4 weeks, their rate of copulation was in the range of 20 to 30%, but no copulation was observed thereafter.

Taken together, these results make it possible to conclude that irradiation of 12 to 24-hour-old pupae with doses of 1750 or 1500 rad significantly reduced their life expectancy while preserving normal geotaxis and mating ability for 3 to 4 weeks after emergence. Subsequently, the aging process clearly accelerated.

DISCUSSION

As far as we know, the literature includes no reports concerning the effect of the irradiation of pupae on the aging of adult *Drosophila melanogaster*. We are therefore obliged to compare our results for irradiated pupae with those obtained after irradiation of adults only.

In the adults which emerged from these pupae, we observed the following:

1) Shorter life span, in agreement with the results of Lamb (1964, 1965, 1978), Nöthel (1965), Atlan *et al.* (1969), Miquel *et al.* (1972), Gartner (1973a, b), Giess and Planel (1977).
2) Dose-effect on the life shortening of mixed populations, also pointed out by Lamb

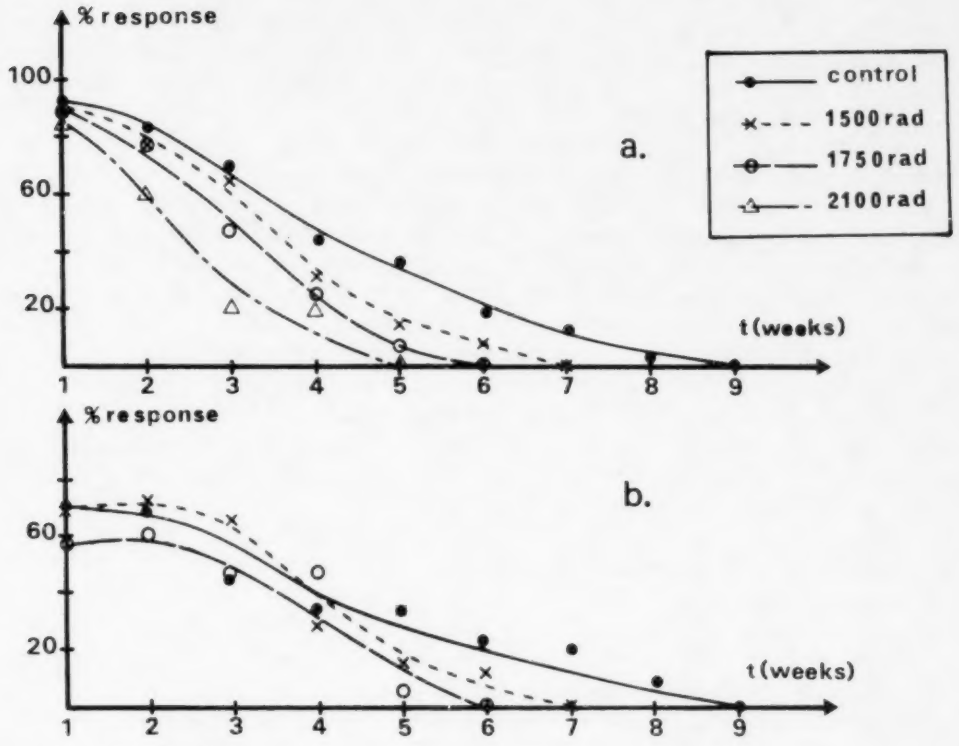

FIG. 6. Behavioral study: responses in relation to age.
a) geotaxis = percentages of males which ascended past the 150 ml mark.
b) mating ability = percentages of virgin males which copulated during the 10 minutes after virgin females were introduced into the vials.

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(1965, 1978) and by Giess and Planel (1977) as well as of virgin or mated males and females. According to Giess and Planel (1977), mated males exhibited no dose effect; however, one such effect is described by Giess *et al.* (1980) for both sexes, including virgin and mated individuals.

3) Greater radiosensitivity in male than female flies. This finding is in agreement with Gartner's results (1973a, b), but seems to disagree with those of Giess and Planel (1977) and Giess *et al.* (1980).
4) Higher radioresistance in virgin than mated animals; this was only noted for males by Giess *et al.* (1980).
5) Induction of sterility in females by the 3100 rad dose. Other authors always reported female sterility for doses from 20,000 to 99,000 rad (Maynard Smith 1958a, b; Lamb, 1964, 1965, 1978; Nöthel, 1965; Giess *et al.*, 1980).

Our findings of reduced longevity in flies, which nevertheless remain fertile, after irradiation of pupae with doses lower than 2500 rad, might be due to the events which occur in 12 to 24-hour-old pupae. At this stage, numerous processes already pave the way for the crisis of metamorphosis in the somatic tissues (for example, epidermal histoblasts pro-

liferation, Poodry, 1980). The gonads, on the contrary, exhibit little differentiation, at least in the females, since the germinal cells are only at the stage of oogonia (Laugé, 1969); these cells constitute a huge reserve which, even when reduced by irradiation, can insure the production of an offspring.

The distinctive life span of the animals treated with 3100 rad, illustrated by their high level of nonemergence and sterility, and by the linear shape of their survival curve, implies that a radiation syndrome combined with the aging process shortens the life of these insects. Several authors obtained similar results after irradiation of adults with high doses (50,000–99,000 rad, Atlan *et al.*, 1969; Miquel *et al.*, 1972, Gartner, 1973b).

On the whole, the present findings indicate that the main effects induced by the irradiation of pupae with doses of 500–2100 rad are the same as those observed in adults irradiated with doses 10 to 30 fold higher. Nevertheless, at least two of the effects produced by the lower doses used in this work were different.

1) The fertility of the treated flies. This effect prevented the lengthening of life expectancy observed by others in irradiated adult females, even for doses in the range of 10,000 to 25,000 rad (Maynard Smith, 1958a, b; Lamb, 1965; Nöthel, 1965). These authors explained the increased longevity they observed by the sterility of the flies. However, we should mention that our results for this particular point are qualitative, since quantitative analysis of the offspring of treated flies was not done.
2) The common rectangular survival curves and the slope values of the regression lines for treated insects compared to their controls suggest that the life of control and treated animals displays a similar evolution.

These two remarks suggest that irradiation of the pupae does not cause important biological damage to the flies. Our behavioral studies confirm this interpretation, since a negative geotatic response as well as mating ability involve motor activity and many receptors (Grossfield, 1978; Ehrman, 1978; Spieth and Ringo, 1983). These process decline with time in both *Drosophila melanogaster* (Herman *et al.*, 1971; Nicolisi *et al.*, 1973; Samis *et al.*, 1981) and *Drosophila virilis* (Aigaki and Ohba, 1984). In our study, the treated flies preserved a qualitatively similar response to that of the control and the shape of their curves was almost the same. Therefore we may justifiably conclude that our results are the consequence of a kinetic change in the normal vital processes which occur during aging and that under certain conditions, the irradiation of pupae allows the process of aging to be distinguished from radiation syndrome.

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EDITORIAL

THE FILES of a journal that has existed for 20 years normally provide an overview of the growth of its chosen subject. Experimental gerontology—the analysis of aging processes with a view to their eventual control in Man—was a topic that, like interplanetary travel, had long appealed to imaginative enthusiasts, but that needed to establish its credentials before it could attract the interest of serious investigators. *Experimental Gerontology* was launched at approximately the point where this could be said to have been done. There had been previous significant research: The work of McCay on dietary restriction, which remains a theoretical landmark, was done in the 1930s. It had, however, to wait for an infrastructure—in comparative biology, in actuarial methods, and in theory—before a discipline could be presented to potential investigators. It had to wait also for the coming together of a group of interested workers who had given the subject some thought.

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An apostolate of this kind already existed when the journal was launched. Those who recall the early meetings of the International Association of Gerontology will recall that they were meeting places for familiar figures. Many of these, who have meantime had the satisfaction of seeing the subject grow (and some of their early formulations revised by new work) are represented on the new, and greatly strengthened, Editorial Board.

The interval between the establishment of experimental gerontology as a serious research topic and the journal's first volume was effectively the age of the tentative unitary theory. Possible contributing factors to the aging process—mutation, error accumulation, limitation in clones, program exhaustion, endocrine factors, and free-radical damage—were listed by different investigators and assessed as candidates for the standing of an overriding age process. Twenty years later one of them may still prove to be such. Many factors, including the new interest in biological effects of radiation, which at that time "kept alive more scientists than it killed," contributed to the ebb and flow of interest in and funding of these various models. One can trace a similar period in many other subjects when they first set into a formal discipline. It is followed then by a period when the published papers shift to specific investigation: Having thought of most of the possible factors likely to be relevant, people go into the laboratory not so much as to demonstrate or disprove theories as to examine the phenomena that need to be explained.

The journal was launched at the point when this seemed ripe to occur, in order to provide a platform for the studies that would emerge from the process. In the late 1960s, NICHD organised, and Leroy E. Duncan Jr. set up a series of annual meetings in which the apostolate of gerontology confronted, and lectured to, an invited audience of the best Ph.D. students we could recruit from U.S. schools. The aim was to set up a cadre of workers in the field (and hopefully shake down a few of potential future Nobel Prize quality) to take gerontology into the laboratory nationwide. Many attendees at those courses now occupy important research posts in the field, have contributed work to the journal,

or both. The confrontation was equally fruitful for the faculty, because the attendees asked searching questions, and established gerontologists received an overview of their colleagues' work, whereas prominent workers in other disciplines were set thinking about the problems that age studies were addressing.

It was said a few years ago that reading the genetic code, although scientifically important, had contributed nothing to clinical medicine. The remark (which was true) was made about a year before the advent of practical genetic biotechnology. One could say the same concerning gerontological research, but one would run the same risk: The reconstitution of the editorial side of the journal in fact takes place when, at least in the field of applied immunology, clinical applications are in sight. The subject has changed in approach: Although one of the unitary theories might still provide us with an overriding point of intervention in the aging of mammals, most of us now think in terms of a multiplicity of clocks, some developmentally coordinated, others, with longer time scales, probably not. The likely importance of brain timing mechanisms has appeared as a product of this view, linking observations such as those on dietary restriction with findings on such topics as DNA repair. The problem in unravelling any such association is the evolved coordination of rate processes within the organism, which causes longevity to chime with reproductive rate, and so on. At the same time, it is clear that the differences in mammalian lifespan, which can be large between related species, are mediated by relatively few genes. We could probably modify the human lifespan now by heroic dietary restriction, unless the McCay effect is confined to overwintering annuals such as small rodents, but the experiment is of low practicability compared with reading back the effects of dietary restriction on immunity, DNA repair, and catecholamine metabolism and intervening at a secondary level.

It is not unreasonable to hope that by the time the journal is again ready for editorial reshaping, clinical papers will have appeared in it. The mode of advent of gerontological intervention in clinical medicine may be through geriatrics (improving the immune status or the disease resistance of the old) but it is perhaps equally likely to involve the attempt to postpone a specific age-related risk (to postpone an anticipated malignancy or degeneration of the normal lifespan, for example). This, rather than 80-year experiments on the prolongation of lifespan by curve-shifting, seems the most likely prediction, unless it becomes possible to identify reliable nonactuarial criteria that could abbreviate experiments on healthy human volunteers. Individual gerontologists who subject themselves to influences such as calorie restriction or antioxidant diets can only provide samples of one, which would be statistically suggestive only if the resulting gain was very large; an unlikely outcome if there are "backup" clocks not coordinated with physiological aging that would eventually strike, but do not do so within the present specific age range.

The decision to reorganize the editorial structure of the journal at this point comes from a number of considerations. One is that though the journal is and was international, attempts to continue production in England with an editor in the United States worked badly, leading to delay and loss of manuscripts. Because I am moving to England to work on a geriatric curriculum for Third World conditions, while returning to the United States to teach medical residents, it would have been unjustified to re-create the former difficulty. A second factor is the undesirability of leaving a journal in the hands of one editor for more than 20 years, and the wisdom of Jefferson's remark that "a man should not too long occupy the same ground." The third is the willingness of the most eminent American experimental gerontologist, Leonard Hayflick, to assume the editorship. Although he was most generous to me as the retiring Editor, I could not have launched or continued this or

any journal by myself. Anything it has done to foster the subject stands to the personal credit of Sir Robert Maxwell, M.C., the founder of Pergamon Press, who not only gave us the opportunity of creating a journal, but who has kept it in print in spite of rising costs and restricted circulation. Much of the quality of the material has been due to the work of the late George Sacher, himself one of the original conspirators of gerontology, the leading researcher of interspecific differences in lifespan and their interpretation, and an irreplaceable loss to the subject.

For my own part I am neither retiring from the subject nor out of touch with it, though I shall not reissue my textbook for the same reasons that I have handed over the journal: Reissue of one man's textbook involves the repetition of one man's ideas, errors and omissions included. The journal has brought me one unexpected pleasure—seeing these errors and omissions corrected by the laboratory work of subsequent gerontologists. One of the chief satisfactions of science is in seeing others answer questions one can't answer oneself. Meanwhile, all of us in the apostolate of gerontology are still engaged with the subject—our deepest satisfaction is that it is now self-powered, and we do not need to push it by hand.

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REVIEW ARTICLE

THEORIES OF BIOLOGICAL AGING

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"The constancy of the *milieu interieur* is the condition for a free life."
Claude Bernard

"The inconstancy of the *milieu interieur* is the condition for the finitude of life."
George Sacher

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BIOGERONTOLOGY, THE study of how and why living organisms age, is a young discipline. Only within the last decade has it attracted the interest of a sufficiently large segment of the biomedical community to be called a seriously studied science. Prior neglect of the field is difficult to explain, if for no other reason than the fact that age changes are one of the few universal properties of living things. Elements that have contributed to the neglect of biogerontology include complex sociological, political, and economic phenomena that influence what is fashionable in science. Of additional importance is the widespread prejudice of ageism, even among scientists, and the consequent negative attitudes of the scientific establishment to a field that, historically, also has been infiltrated by pseudoscientists and outright charlatans.

It is not surprising, therefore, that the field attracted few young scientists and was a focus of derision by senior scientists whose negative attitudes toward gerontology kept it in a primitive state of development. Now much of that has changed. Nevertheless, the relative newness of the field, with the consequent lack of a strong database, has encouraged speculations on the theoretical underpinnings of the field and is one of two fundamental reasons why theories of biological aging abound. An additional cause of the plethora of biogerontological theories is that manifestations of biological changes over time affect virtually all components of living systems from the molecular level up to that of the whole organism. These hierarchical changes have made it possible to construct theories of aging based on events that occur over time at the level of the molecule, organelle, cell, tissue,

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organ, or whole animal. Depending more upon the bias of the theorist than actual fact, a selected age associated change within this hierarchy will often be defended with great vigor and emotion. Nevertheless, most current theories of biological aging suffer from the criticism that they are, or may be, mere expressions of the effect of some more fundamental change. There is common failure to realize that changes more fundamental to the one observed may induce the effect that was chosen for study.

The late George Sacher, a leading gerontological theoretician, has referred to theories of this kind as "aspect theories" and laments the common practice of transforming the changes found in one narrow aspect of the plethora of age changes into a generalized theory of universal applicability.

Currently popular aspect theories include perturbations in the following biological systems or phenomena as a principal cause of aging: the immune system, the neuroendocrine system, somatic cell mutation, the genetic program, error accumulation and repair, selected physiological states, generation and scavenging of free radicals, cross-linking or instability of molecules, changes in entropy, lipofuscin accumulation, and cell loss.

Even more naive is the common practice of aspect theorists wrongfully to conclude or infer that the aspect studied is unicausal. The preponderance of evidence in gerontology is strongly persuasive that those attributes of biological instability that by common belief are considered to be age changes have a multiplicity of causes. This does not exclude the possibility that the fundamental cause of aging may be the pleiotropic expression of, for example, a single class of information-containing molecules. Such a finding could qualify as the fundamental "cause" despite having the character of producing numerous secondary and tertiary changes.

The following discussion considers the most popular current theories of biological aging. Each has one or more strong advocates, a body of data that lends support for the hypothesis, and some evidence that may undercut the theory as universally applicable. Taken together, most of these theories are not mutually exclusive. All or some may operate simultaneously. Finally, it should be stressed that the current popularity of these theories should not be interpreted to mean that another theory of aging, yet to be described, is not closer to the truth. The theories have been grouped in respect to one or more common elements but otherwise are presented in no particular order.

ORGAN THEORIES

Organ theories of aging are based on the common finding that with age, increments or decrements occur in organ function. The organs currently most favored as the likely ultimate origin of all age changes are the immune system and the brain or neuroendocrine system.

1. The immunological theory of aging

This theory rests on two major findings, details of which can be found in reviews by Makinodan and Kay (1980), Weksler (1981), Kay (1979), and Walford *et al.* (1981a). First, with age the ability of the immune system to quantitatively and qualitatively produce antibodies diminishes. The thymus dependent system reveals the greatest decline.

Second, as normal immune response declines, autoimmune manifestations increase greatly with age, and the immune system becomes less able to discriminate between self and nonself, resulting in an increase in autoimmune diseases. Thus, if the immune system

becomes less efficient over time, the ability of an individual to successfully thwart the effects of microbial pathogens or opportunists diminishes. An increase in autoimmunity would, similarly, result in an increase in chronic autoimmune diseases that are characteristically associated with aging. The consequences of both are an increased vulnerability to disease and pathology.

The master genetic control of the immune system is the main histocompatibility complex (MHC). Smith and Walford (1977) found that 90th percentile survivorship (which is an approximate measure of the maximum lifespan potential) occurs in 14 different strains of mice genetically identical except for alleles at the MHC. It has been suggested that the MHC may be one of the gene systems controlling aging (Walford, 1979). The genes regulating superoxide dismutase (SOD), mixed function oxidase, and the level of cyclic nucleotides are on the same chromosome and are genetically linked to the MHC (Walford *et al.*, 1981a). Walford *et al.* (1981b) points out that SOD and the mixed function oxidases protect against damage by free radicals that may cause age changes (See *Free radical theory* following) and that cyclic nucleotides are involved in cell differentiation and proliferation. He suggests therefore that the genetic linkage of all these factors on chromosome number 6 in humans and number 17 in mice, where the MHC is also found, makes these chromosomes the focus for age changes (Walford, 1980).

The immune response peaks at about the time of puberty in mammals and then declines in old age to about 5% or 10% of its youthful value (Walford *et al.*, 1981b). As this decline occurs the incidence of autoimmune manifestations increases. Simultaneously, the appearance of autoantibodies in mice and humans shows a dramatic increase with age. Immune dysfunction with aging is generally manifested by a decline in the response of lymphoid cells to immunological stimuli, such as reaction to an alloantigen or mitogen (Walford *et al.*, 1981a).

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There is good evidence that the decline in immune function is due to an age associated deficit in the T cell component of the immune system. Thymic involution precedes, and therefore may be responsible for, the age-dependent decline and the ability of the immune system to produce competent T cells (Kay, 1979). Thymic atrophy may therefore be the trigger for immunological aging and, in the view of some, act as the master chronometer (Walford *et al.*, 1981a).

Recently, Walford *et al.* (1981b) demonstrated that the number of population doublings that cultured human peripheral T cells will undergo declines as a function of T cell donor age. This is the first demonstration of a finite limit on the replicative ability of normal human T cells and the inverse relationship of that replicative capacity to donor age. It extends to yet another cell type the same finding originally made by Hayflick (1965), and Hayflick and Moorhead (1961) with cultured normal human fibroblasts, a finding that now appears to be universally applicable to all normal cells.

Advocates of the immunological theory of aging also argue that most of the diseases of old age or the late-life, high incidence afflictions are characterized by some degree of immune dysfunction. These diseases include cancer, maturity-onset diabetes, senile dementia, and several vascular diseases (Walford *et al.*, 1981a). It has also been reported that the HLA antigens B7 and Cw 3 occur at about twice the frequency in senile dementia of the Alzheimer's type as in the normal population (Walford *et al.*, 1981a).

Despite the innate appeal of immune system changes as the master chronometer of aging, the theory suffers from faults applicable to other organ-based theories. First, the immune system as a cause of all age changes lacks universality. That is, many organisms

that age lack an immune system, or at least the kind of immune system found in higher vertebrates from which most of the aforementioned data have been obtained. Second, the immune system is closely subject to regulation, for example, by hormones (Fabris, 1977). As such, immunological dysfunctions could have a more fundamental cause, rooted, for example, in the neuroendocrine system, whose activities are clearly influenced by the age of an individual. This is a good example of the difficulty in distinguishing cause from effect. Are the causes of all age changes due to decrements in the immune system? Or are those decrements merely reflections of the effect of more fundamental causes of immune dysfunction, such as hormonal or neural effects? Third, all cells, including those composing the immune system, probably undergo age changes that are genetically based. Why then should the immune system be emphasized as the basic cause of age changes when virtually all cells in most organ systems incur comparable functional decrements with time? The increase in autoimmune phenomena with age may not be attributable necessarily to decrements in the immune system but to actual antigenic changes in the target cells themselves as they age. For instance, the increase in cross-linking of collagen and other protein molecules (see the following for a separate discussion of this theory) may produce novel antigens that provoke the same kind of antibody response in an old individual as is produced by the presence of, for example, a new viral antigen in a young individual.

It is equally plausible to argue that several age associated diseases themselves cause the aforementioned immunological deficits. Age changes then may be the result of decrements occurring in virtually all cells and at different rates. Clearly the immune system changes with age, and it produces a plethora of immunological dysfunctions. Nevertheless, its role as the fundamental cause of all age changes is unproven.

2. The neuroendocrine theory

Like adherents of the immunological theory, adherents of the neuroendocrine theory argue that decrements in a system so essential to survival and homeostasis as neurons and hormones must play a profound role in the regulation of age changes. No part of the body acts in isolation from neurological and endocrine systems. Perturbations in these systems resulting from changes occurring over time should have important and widespread effects. If, for example, decrements occur in the hypothalamus, pituitary function would be affected, and this in turn would have a cascading effect on many endocrine target cell functions. As one might expect, there is a veritable catalog of age-related changes in cell functions reported to be associated with changes in hormones or neural factors (Schimke, 1980). These changes may be the necessary predisposing conditions for age-associated diseases. For example, the steroid loss at menopause may lead to the conditions necessary for osteoporosis.

The loss of neurons and endocrine cells with age has been recognized for many years, but the role that this loss may or may not play in aging remains a subject for considerable speculation. Neuronal loss occurs in selective areas of the brain (Brody, 1980). Decrements in motor function, sleep, behavior, and cognitive function could result from loss of monoaminergic neurons in the locus ceruleus and substantia nigra, and of cholinergic and other neurons in the hippocampus, caudata putamen, and cerebral cortex. Age-associated neuronal cell losses in the critically important hypothalamus and limbic system and in the autonomic centers are not well studied; nevertheless, several studies conclude that a 10% decrease occurs in total brain weight with age (Brody, 1980). This loss may not necessarily reflect cell loss but may include loss of fluids and/or ground substance. The cell loss that

does occur is generally restricted to discrete areas of the cerebral and cerebellar cortex (Brody, 1980). One particularized neuroendocrine theory of aging has been postulated by Denckla (1974). He proposes that evidence exists for a new pituitary hormone called "decreasing oxygen consumption hormone" (DECO), which emerges at puberty in mammals under stimulation by thyroid hormones. In the rat, DECO appears to be secreted at continuously high levels throughout its life span. However, with age, the rate of secretion decreases. DECO, it is argued, blocks the responsiveness of peripheral tissues to thyroid hormones with advancing age. It is also assumed that DECO is responsible for the loss of growth hormone responsiveness. Because growth and thyroid hormones govern protein synthesis and cell division, DECO could indirectly provoke widespread deleterious effects. It has been called a "death hormone." Although Denckla presents some evidence for the existence of DECO, that evidence is not persuasive, and there is considerable reluctance to accept the existence of DECO, to say nothing of its putative role as a master chronometer. Furthermore, the postulated hormone does not affect the many other age-associated deficits that are not orchestrated by the pituitary.

Many age-associated response differences to hormones are known. Some are due to changes in receptor concentrations (Roth, 1980), and others reflect disturbances in hormone regulation that lead to a progressive increase in the adaptive latent period of enzymes (Adelman *et al.*, 1980).

Like the immune theory of aging, the neuroendocrine theory lacks universal applicability. First, not all organisms that age have complex neuroendocrine systems. Second, the age-associated deficits found in the neuroendocrine system may be the result of basic changes that occur, for example, in the genome of all cells as they age. Consequently, there would be no reason to select the neuroendocrine system, or any other single system, as the primary orchestrator of age changes. All organ systems age, and most likely at different rates. Once again we are obliged to distinguish between cause and effect.

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PHYSIOLOGICAL THEORIES

1. The free radical theory

Denham Harman, chief advocate of this theory states ". . . aging is largely due to free radical reaction damage . . ." (Harman, 1981, p. 7125). Free radical reactions arise from (a) exposure of cells and their organelles to ionizing radiation, (b) nonenzymatic reactions and, (c) enzymatic reactions, particularly in photosynthesis and the reduction of O_2 to water. Harman emphasizes that the theory is largely applicable to mammalian aging, where O_2 is the main source of damaging free radical reactions.

Free radicals are atoms or molecules bearing an unpaired electron. Free radicals are ordinarily very reactive because of the tendency of electrons to pair. Moreover, because the reaction of a free radical with a stable molecule produces another radical, such events often result in chain reactions in which a single free radical initiates a process that consumes many stable molecules. The oxidation of polyunsaturated fatty acids, such as occurs in the phospholipids of biological membranes, readily occurs by such a free radical chain reaction (Pryor, 1978). In respiring cells one source of free radicals is the univalent pathway of reduction of dioxygen, which generates the superoxide and the hydroxyl radicals (Fridovich, 1983). Free radical oxidation of polyunsaturated acids gives rise in vitro to a host of aldehyde and hydrocarbon products. Among the latter are ethane and pentane, and these hydrocarbons can be measured in the breath of experimental animals and hu-

mans (Lawrence and Cohen, 1982). Moreover, situations expected to impose an oxidative stress or to lower the levels of the antioxidant defenses result in enhanced expiration of ethane and pentane. This suggests that free radical oxidation of polyunsaturated fatty acids can occur in vivo.

A lipoidal fluorescent pigment called lipofuscin, or age pigment, has been seen to accumulate in nondividing mammalian cells (Shimasaki *et al.*, 1977). A similar substance can be produced in vitro by the polymerization of proteins or peptides with malondialdehyde (Chio and Tappel, 1969). Malondialdehyde is one of the products of the free radical oxidation of polyunsaturated fatty acids.

Because free radicals are highly reactive, all cells are susceptible to damage caused by them and on a random basis. The damage produced could be expressed as (a) cumulative oxidative alterations in collagen, elastin, and DNA itself, (b) breakdown of mucopolysaccharides through oxidative degradation, (c) accumulation of metabolically inert substances such as ceroid and age pigments by oxidative polymerization reactions, (d) changes in membrane characteristics of mitochrondria and lysosomes, and (e) fibrosis of arteriols and capillaries secondary to vessel injury by products resulting from peroxidation of serum and vessel-wall components (Harman, 1981).

Free radical damage can be thwarted by antioxidants such as α-tocopherol, heme-containing peroxidases, selenium-containing glutathione peroxidase, superoxide dismutases, and by DNA repair mechanisms. The major evidence for a free radical role in aging is the effect of feeding antioxidants to laboratory animals. This evidence is only indirect because there is no direct evidence that free radicals themselves are being modified. Dietary antioxidants increase life expectation in mice, rats, fruitflies, nematodes, rotifers, and neurospora. In mice, increases in life expectation of up to 30% have been observed when dietary antioxidants are administered (Harman, 1981). It is important to note that life expectation increased but not maximum life span. That is, more animals reached a higher average age before death when fed antioxidants, but the maximum life span of the species was unaffected.

Sohal, in studies using insects, has shown that life span is a function of total oxygen consumption. When the rate of oxygen consumption was reduced by lowered temperature, by restricting the level of physical activity, or even by sublethal irradiation, the life span was increased (Sohal and Buchon, 1981). Because oxygen reduction is an important source of free radicals, such data may be taken as support for the free radical theory of aging.

Harman believes that there is a single basic cause of aging and that "the aging process may be simply the sum of the deleterious free radical reactions going on continuously throughout the cells and tissues" (Harman, 1981, p. 7127). He provides data for the role of free radicals in cancer, cardiovascular diseases, degenerative diseases of the central nervous system, and depression of the immune system (Harman, 1981). Unlike organ-based theories of aging, the free radical theory, because of its cellular basis, has the appeal of potential universality.

The experimental data suggest that administration of free radical antagonists, such as antioxidants, increases life expectation substantially. Nevertheless, factors that tend to increase life expectation do not necessarily play a role in the aging process. For example, from 1900 to the present day there has been an increase in life expectation at birth in humans in the United States from 47 to about 73 years (U.S. Department of Commerce, Bureau of Census, 1982). Most of this increase resulted from the virtual elimination of infectious diseases as a cause of death, especially in the younger age groups. This has oc-

curred substantially from the development of virus vaccines, discovery of antibiotics, and improvements in hygiene. Few would argue that microorganisms cause aging. Antioxidants may simply inhibit or delay the expression of some diseases just as vaccines and antibiotics do. All contribute to an increase in life expectation but not to an increase in life span. However, in one experiment by Comfort (1979) both the mean and maximum life span of C_3H mice fed the antioxidant ethoxyquin increased significantly (Kohn, 1978). Comfort (1979) speculates, however, that ethoxyquin may have limited the toxicity of the standard laboratory diet or that antioxidants prevent the oxidation of essential nutrients in the diet. The action of antioxidants, therefore, may be to somehow improve the diet with no direct intracellular effect on putative free radicals. This is supported by the fact that in most experiments where antioxidants are fed, a reduced body weight results when compared with that of controls. This occurs presumably because the presence of the chemicals makes the food less palatable or affects digestion or food absorption. Undernutrition or calorie restriction is a well known means of increasing the life span of animals (McCay *et al.*, 1939) and antioxidant feeding experiments may be mimicking aspects of that phenomenon.

The causes of death appearing on the death certificates of old people must be distinguished from all the normal physiological decrements that occur with time and that increase vulnerability to the ultimate cause of death. Antioxidants inhibit the development of some cancers, and, according to Harman (1981, p. 7124), free radical reactions are "strongly implicated in the disorders of the cardiovascular and central nervous systems." These diseases are the leading causes of death in most developed countries.

Selenium, a component of the antioxidant glutathione peroxidase, is thought to be the cause of low incidences of cancer in several geographical areas (Shamberger, 1976). Immune responses are thought to be enhanced by adding antioxidants to the diets of mice (Harman, 1981). Antioxidants are reported to slow development of amyloidosis and autoimmune disorders and to inhibit prostacyclin synthetase, which is a potent inhibitor of platelet aggregation (Harman, 1981). Prostacyclin is thought to contribute to atherosclerosis and is also a powerful vasodilator (Harman, 1981). Age pigment formation, thought to inhibit cell function and discussed in the following as a separate theory, is slowed by antioxidants (Tappel *et al.*, 1973). Free radicals are also thought to be involved in the formation of the neuritic plaques associated with senile dementia of the Alzheimer type (Harman, 1981), although the effect of antioxidants on amelioration of this disease is not known.

Even if this catalog of ailments was to be increased and the cause of all attributed with certainty to the action of free radicals, it would not constitute evidence that free radicals cause age changes for the same reasons that infectious diseases, poor hygienic conditions, automobile accidents, or use of handguns do not produce age changes. The amelioration of some, or all, of these diseases or causes of death by antioxidants simply increases life expectation, not life span. It is probable that only by increasing life span, or maximum age of death, of members of a species, will important insights be made into the aging process. Free radical damage as a cause of age changes is an attractive idea with substantial support for its effect on disease states and life expectation, but its role as a fundamental cause of age changes is doubtful.

2. *The cross-linkage theory of aging*

This theory focuses on molecular changes that are known to occur with age in substances within the extracellular compartment and, intracellularly, in information-containing mol-

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ecules such as DNA and RNA. The importance of the extracellular compartment may be appreciated by observing that 23% of body weight is contained in the extracellular compartment (Kohn, 1978). The major proponents of the cross-linkage theory are Kohn (1978) and Bjorksten (1974).

The theory holds that age changes result when two or more macromolecules become linked covalently or by a hydrogen bond. Such linkages, said to be reversible, accumulate over time. Molecular aggregation and immobilization increases, and the resulting inert or malfunctioning molecules accumulate and become increasingly resistant to catabolic processes. DNA may thus become damaged, leading to mutation or cell death. Irreplaceable molecules become reduced in numbers, and intracellular transport may be impeded. Progressive reduction of space available for vital processes also occurs. Nonremovable cross-linked aggregates may "clog" glandular cells, thus impeding critical production and release of hormones and other cell products. Cross-linking in molecules such as collagen could decrease solubility, elasticity, and permeability. This would increase viscosity in the extracellular compartment, thereby impairing the flow of nutrients and waste products into and out of cells. These events are believed by the theory's advocates to result in all of the normal age changes observed at higher levels of organization. Cross-linking, they believe, is the first molecular event leading to most age changes.

At least for the collagen molecule an increase in cross-linkages over time has been demonstrated to almost everyone's satisfaction (Kohn, 1978; Verzar, 1956; Bjorksten, 1971). There is also evidence for the occurrence of cross-linking over time in other proteins and in DNA (Bjorksten, 1971). Bjorksten (1971) lists an impressive number of naturally occurring known cross-linking agents to be found in living tissue: aldehydes, sulfur cross-linkages, alkylating and acylating agents, quinones, free radicals, antibodies, polybasic acids and their esters, citric acid, polyvalent metals, and cross-linkages formed as integral parts of reacting molecules such as conversion of specific lysine residues to aldehydes, which react to cross-link collagen. Bjorksten also argues that because free radicals are effective cross-linkers, the free radical theory is simply a special case of the more general cross-linkage theory (Bjorksten, 1974).

The cross-linkage theory has in its favor, first, evidence that the phenomenon does indeed occur and, at least for some molecules, linkages do accumulate over time. Second, the phenomenon has a known, or theoretically probable, physiological basis, and finally, it is potentially universally applicable because the phenomenon occurs in molecules containing nucleic acids.

Criticisms are based on the likelihood that many of the vital molecules that may be cross-linked do indeed turnover. Opponents argue that only nonrenewable substances age, and continuously renewable cells do not age. Nevertheless, most collagen does not turnover, and renewable cells still may age because the genome that gives rise to them may itself be imperfect and accumulate damage or imperfections in cells that are passed on to their progeny at each subsequent division cycle.

Of greater importance is the criticism that regardless of the qualitative or quantitative magnitude of cross-linking that has been described, it has not been demonstrated directly that it is sufficient and necessary to produce the biological decrements of aging attributed to it.

Finally, it has not been proposed how the rate of molecular cross-linking is governed to account for the great diversity in life spans of animals. How, for example, would one ac-

count for the occurrence of cross-linking in the same class of molecules at a rate 30 times faster in mice than in humans?

3. Waste-product accumulation

Pigmented inclusion bodies in nondividing cells such as neurones and those found in skeletal and cardiac muscle have been known to accumulate with age in many animal species since first observed in 1911 (Stübel, 1911). These inclusion bodies represent aggregated waste products that accumulate to the point of suggesting that they may impede cell function and cause age changes. The distribution and characteristic yellow-green to orange fluorescence of these pigments have been well documented (Hamperl, 1934). (The pigments are yellow-brown when observed by light microscopy.) The inclusions often have been found near the Golgi apparatus (Bondareff, 1964), but it is unclear whether this has functional significance or is merely coincidental. Suggestions that the inclusions may represent degenerated mitochondria (Hess, 1955) or products of lysosomes (Gedigk and Bontke, 1956; Essner and Novikoff, 1960) or the endoplasmic reticulum have also not been proved.

The age-associated pigments are now more frequently referred to as lipofuscin. They probably occur as a result of auto-oxidative reactions (Tappel *et al.*, 1973) because extremely fluorescent material forms when amino acids react in vitro with malondialdehyde, a product of lipid auto-oxidation. Vitamin E deficiency increases deposition of lipofuscin, but supplementation of vitamin E does not inhibit lipofuscin accumulation or extend longevity. Others (Nandy and Bourne, 1966; Spoerri and Glees, 1974) report that centrophenoxine (dimethyl-amino-*p*-chlorophenoxyacetate) reduces lipofuscin concentration. Strehler *et al.* (1959) found that the volume occupied by lipofuscin granules in human myocardium increases linearly with age. The rate of accumulation is about 0.3% of the total heart volume per decade or about twice that amount in intracellular volume per decade. At 90 years of age as much as 7% of the intracellular volume may be occupied with lipofuscin granules. Lipofuscin granules contain cathepsin, acid phosphatase activity, unsaturated lipids, various enzymes, and chromophores. In addition, age pigments react positively in tests for proteins, lipids, and carbohydrates, thus implying extreme complexity. Lipofuscin is highly cross-linked and is most likely the result of accumulation of auto-oxidation of lipid components of lysosomes and other membranous cell organelles. As much as 25% of the intracellular volume of neurones in 28-month-old rats and mice contains age pigments. Large neurones in centenarian humans may be almost totally (75%) occupied by lipofuscin except for the nonfluorescent nucleus (Treff, 1974; Strehler, 1977).

More details on the origin, accumulation, and location of lipofuscin may be found in several reviews (Strehler, 1964, 1977; Bourne, 1973; Toth, 1968).

The accumulation of waste products in aging cells as a cause of age changes has some intuitive appeal; nevertheless, there is inadequate evidence to substantiate this hypothesis. The seemingly random distribution, rate of accumulation, and lack of universal occurrence in significant numbers and kinds of cells imply a secondary role at best for the accumulation of age pigments as the fundamental cause of age changes. The facts that (a) vitamin E deficiency increases lipofuscin deposition without accelerating other age changes and (b) vitamin E administration does not prolong life span or decrease lipofuscin accumulation also argue for its minor role as a cause of significant changes.

It is unclear whether lipofuscin granules actually damage cells by impeding function.

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Accumulation in modest amounts in neurons is not known to interfere with neuronal activity.

GENOME BASED THEORIES

There is a general belief that longevity is determined to a large extent by genetic mechanisms. In humans, for example, the mean difference in longevity between fraternal twins was found to be twice as great as that in identical twins (Kallman and Jarvik, 1959). The ancestors of centenarians and nonagenarians were found to have significantly greater longevity when compared with a series of ancestors of individuals not selected for great longevity (Pearl and Pearl, 1934). In many animal species the female is more longevous than the male, but this is by no means true for all animal species (Comfort, 1979). In a study of inbred mouse strains Goodrick (1975) estimated that half of the variance associated with longevity was due to genetic factors. Heterosis, or hybrid vigor occurs when members of two different inbred strains are themselves mated. They produce F_1 hybrids having greater longevity than either parental strain. Finally, a phenomenon known as the Lansing Effect suggests that in some animals, including humans, the progeny of older mothers have a shorter life expectation and that this effect may extend through several generations (Strehler, 1977; Comfort, 1979).

1. The somatic mutation theory

The somatic mutation theory of aging enjoyed its greatest popularity in the late 1950s and early 1960s as a derivative of burgeoning developments in the field of radiobiology. The central concept is that the accumulation of a sufficient level of mutations in somatic cells will produce physiological decrements characteristic of aging. If mutations are the fundamental cause of age changes, they must occur randomly in time and location (Maynard-Smith, 1962). Early champions of this idea were Szilard (1959) and Failla (1958, 1960). Failla postulated dominant mutations as causes of aging. Szilard argued that aging was due to genes ("targets") being "hit" or "struck" by a mutational event, which unlike Failla, he regarded as recessive. Thus a pair of homologous genes must be hit at a particular rate and in a sufficient number of cells in order to achieve phenotypic expression.

Maynard-Smith (1962) pointed out that if Szilard was correct, inbred animals, homologous at most gene loci, would display the maximum species life span because homozygous faults would be lethal and heterozygous faults would be few or nonexistent. Yet in mice and drosophila, inbreeding reduces life span. Furthermore, Szilard's hypothesis would predict that diploid organisms would live longer than their haploid counterparts who contain only one chromosome set. In the hymenopteran wasp, *Habrobracon*, haploid and diploid males have identical life spans. Haploid males are more sensitive to ionizing radiation than are diploid male wasps, yet irradiation shortens the life span of diploids far more than that of haploids. These observations are inconsistent with the mutation theory. Although reduced life spans do occur in irradiated animals, extended life spans have also been observed (Sacher, 1963; Lamb, 1964). Also, irradiated old animals should show accelerated age changes as should animals treated with mutagenic agents, but they do not (Alexander, 1969).

Curtis, the last major advocate of the somatic mutation theory, based his conclusions on the frequency of abnormalities observed in the chromosomes of dividing cells in the livers of old mice (Curtis and Miller, 1971). He found a higher frequency of abnormalities

in the cells of a short-lived strain when compared with those found in long-lived strains of mice. Curtis made similar findings in guinea pigs and dogs. Nevertheless, other comparisons between short and long-lived strains were inconsistent with these findings and hybrids between short and long-lived strains did not yield the expected results. Neutron irradiation of dividing cells was found by Curtis to yield aberrations in up to 90% of the cells, yet life span was unaffected.

In the past decade few significant studies have been conducted on the role of somatic mutations in aging. In spite of the contrary evidence cited above, there is an expectation that the critical experiments should be redesigned using the technology of modern molecular biology.

2. *The error theory*

This theory, to some extent derivative of the somatic mutation theory, was first postulated by Medvedev, (in a Russian journal, n.d.) elaborated by Orgel (1963) and Medvedev (1972), and received experimental support principally by Holliday and Tarrant (1972).

It has been suggested that the repeated DNA nucleotide sequences in the genome of eukaryotic organisms may be (a) a reserve of information for evolutionary change, (b) a means of increasing functional expression and (c) a reserve mechanism for protecting vital information from random errors that may occur in functioning DNA sequences. Medvedev proposed that the loss of unique, nonrepeated DNA sequences could produce age decrements and that selected reiterated sequences may be an evolved means for delaying the inevitability of the event by providing redundancy necessary for the maintenance of vital information. Cutler (1974) has provided equivocal experimental evidence in support of Medvedev's view.

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Derivative of error accumulation in reiterated DNA sequences is the notion of Orgel (1963), in which he postulates the occurrence of inaccuracy in protein synthesis as the essential source of age-associated decrements in cell function. This hypothesis resulted in a flurry of experiments designed to learn whether an incorrect amino acid incorporated in a protein molecule could accelerate aging phenomena or whether misspecified proteins accumulated in old cells. Errors in enzyme molecules that processed information-containing molecules were thought to be the most important potential sources of important damage. A misspecified enzyme could produce a cascade of faulty molecules with presumably profound effects called an "error catastrophe." Holliday and Tarrant (1972) claimed to have obtained evidence for the error catastrophe theory, but many other studies have provided no evidence for its support. The idea is now in general disfavor despite the fact that altered proteins are frequently found in the cells of old organisms.

A correlate of error accumulation as a cause of age changes is the effectiveness of those systems that repair genome damage. Hart and Setlow (1974) obtained evidence from the cultured cells of several different species that the efficiency of repair of ultraviolet damage to DNA was directly correlated to species' life span. Again, contrary evidence has also been reported, and the original finding currently is being re-assessed.

3. *The program theory of aging*

Adherents of this theory, unlike advocates of such stochastically based theories as error accumulation, postulate a purposeful sequence of events written into the genome that

leads to age changes much as similar instructions written into the genetic message lead to the orderly expression of developmental sequences.

The conceptual simplicity of this idea is part of its attractiveness, but the attempts to test it experimentally have met with little success. The discovery by Hayflick and Moorhead (1961) that cultured normal human and animal cells have a finite ability to replicate and function has provided the best evidence in support of the theory, but so does the fundamental fact that aging occurs naturally in intact animals. Programming assumes an orderly sequence of events with which few would disagree, but it does not provide mechanistic details. On the other hand, it has been effectively argued that although events occurring from conception to the full expression of adulthood may be programmed, subsequent events characterized as aging may not be purposely determined by the genome. That is, age changes may be produced by a kind of free-wheeling, nongenome dependent continuation of the inertia developed from previously determined developmental events. Therefore, function declines or terminates in a more or less random fashion similar to the eventual demise of a new automobile that is poorly repaired or maintained.

To complete the analogy, the manufacture of an automobile, like the growth of an individual animal, is predicated on the presence of accurate blueprints and their proper execution. What happens after the automobile is built or after the individual reaches sexual maturation is not governed by blueprints, but occurs in most systems randomly and inevitably. Which system fails first and leads to the demise of the automobile or individual is, therefore, a random process with, nevertheless, a narrowly expressed "mean time to failure." This would be characteristic of the specific brand of automobile or particular animal species. Regarded in these terms, aging in its extreme manifestations occurs only in humans or the animals we choose to protect, such as domestic or zoo animals. The survival of a species does not depend on its members surviving much beyond the age of sexual maturation. From the standpoint of evolution there is no survival value for our species to have a life expectation much beyond the reproductive years.

In terms of modern physics, a genetic program should succumb to the second law of thermodynamics, which states that a closed system tends to a state of equilibrium or of maximum entropy in which nothing more happens. That is, ordered systems tend to move to greater disorder. The initially well organized genetic program, by increasing entropy, thus becomes disordered, producing those changes recognized as aging. Our mortality may, in this way, be decreed by the second law of thermodynamics. Although this may be a tenable hypothesis as it pertains to somatic cells or to individual members of a species, it seems to fly in the fact of the enormous amount of evidence for biological evolution that superficially appears to be in conflict with the second law. Moreover, it seems to be in conflict with the apparent immortality of the germ plasm and certain immortal, abnormal, cancer cell populations. Medawar (1957) has argued persuasively that the presence of deleterious genes in a species might be thwarted by a selection process that would postpone its manifestations if it were not possible to eliminate it. This strategy would result in the piling up of deleterious genes in the post-reproductive period when their expression would do less harm. A variation on this theme is expressed by Williams (1957), who postulates pleiotropic genes having both favorable and unfavorable actions. If the favorable gene expression is able to increase fecundity, that gene might be selected even though it might express a deleterious action later in life. Deleterious age changes then would be the penalty paid by individuals for the expression of beneficial genes early in life. An accumulation of such late-acting genes in various organ systems would behave like late-programmed events

and give rise to the entire constellation of age changes. Sacher is critical of the program theory of aging for what he believes to be errors in logic (1968, 1982). He illustrates his point by comparing the life histories of annual plants and mammals. Annual plants are semelparous, that is, they are characterized by a single reproductive effort, completed at the end of the life span, and frequently not until somatic cell death. The final step of the reproductive process, seed dispersal, depends on death of the plant, and this requires that senescence be closely integrated with prior stages. The stages are known to be under the specific control of hormones and end with formation of specific hydrolytic enzymes. Sacher restricts programmed aging to cases such as this where there is specific control of onset either by internal or external signals, presence of a specific enzyme mechanism, and finally, a functional role for senescence and/or death in a specific temporal relationship with other life processes. The rapid aging and death of the Pacific salmon after spawning is a good example of this event occurring in animals.

Mammals, on the other hand, are examples of iteroparous reproduction, where reproductive success depends on producing a number of litters over an extended reproductive span. Sacher argues that this offers no functional role for senescence and death. On the contrary, he maintains that this would place a premium on the maintenance of physiological vigor and survival. Long life in mammals, therefore, is the result of selection for an extended period of assured physiological performance. A great whale that lives 30 times longer than a mouse has a million times more cells at risk for age changes. Nevertheless, a comparable whale cell is orders of magnitude more stable than a mouse cell. "It would be expected," says Sacher (1982, p. 350), "that the selective process acts on mechanisms for *increasing* the stability of the organism at all levels, from the molecular to the systemic." Sacher emphasizes the more evolutionary logical role of genetic systems that maintain life rather than suppositions that these systems might program age changes. Until it can be shown that evolution selects for greater longevity, at least in mammals, the study of life maintenance systems or "longevity assurance genes" may be more productive than the current emphasis on a search for the causes of age-associated physiological decrements (Sacher, 1968, 1982).

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Dedication—This paper is dedicated to the memory of George Sacher and Robert Kohn, whose recent premature deaths have deprived biogerontology of two of its leading theoreticians.

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THE COMPARATIVE BIOLOGY OF LONGEVITY AND LIFETIME ENERGETICS

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Abstract—The relationships between longevity and cumulative energy metabolism in homeothermic vertebrates are explored interspecifically as allometric functions, and intraspecifically in terms of metabolic extension, through restriction of energy intake, hibernation, and torpor. A new equation for marsupial longevity is presented. The first evidence of age-dependent mortality in a population of hummingbirds is shown. Hummingbirds are seen to be model subjects for the study of longevity in nature.

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INTRODUCTION

EVIDENCES OF the aging process are generally first detected in humans at about 30 years of age, when decline in physiological functions can begin to be quantified. Subsequently, the remaining capacity in metabolic, pulmonary, cardiovascular, and renal performance can be expressed as percentage of "initial" function at age 30, before the declines begin (Mildvan and Strehler, 1960; Shock, 1977). Cutler (1983) observed: ". . . the reason why humans begin to show significant signs of aging if they live much beyond 30 years is because there was normally little chance for humans to live much past that age, so there was no need to postpone aging any further. It was not because of the impossibility of further life span."

Nevertheless, these declines in functions indicate that there are finite limits. These must have been incorporated in the evolution of workable life histories. If we are to develop rational responses, medical or sociological, to the phenomena of aging and finite longevity, we need to understand the biology that predated our biomedical culture. The comparative approach has been used effectively by Cutler (1978) to synthesize a biological view of the aging process. Much insight may remain to be gained by further comparative studies, helping to insure that gerontological research is not unduly restricted in scope by inadequate background information. It seems worthwhile to review some aspects of comparative longevity.

Allometry provides a useful comparative framework to organize facts into instructive patterns by utilizing the dominating quantitative influence of body size in interspecific correlations with life history variables. Maximum reported longevity (t_{max}, y) has been related to body mass (M, kg) by Sacher (1959):

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for captive eutherian mammals ($r = 0.77$) : $t_{max} = 11.6\ M^{0.20}$ (1)

The positive fractional exponent 0.20 is the ratio in powers of 10 for the increase in life span to body size; a 10-fold increase in longevity being a consequence of a 100,000-fold increase in mass (as from mouse to deer sizes). However it is well known that our species enjoys a life span considerably longer than other mammals of our size. Equation (1) predicts a maximum lifespan of 27.1 years for our 70 kg size, but this span is completed before our actual physiological decline begins (Shock, 1977; Mildvan and Strehler, 1960). Few wild animals are thought to survive environmental hazards of predation, disease, and accident, so that aging is not often observed in wild populations (Cutler, 1983). However, there are indications that the search has not been adequate, in terms of numbers of animals or length of study (Botkin and Miller, 1974; this study).

In this paper, I reexamine the hypothetical relationships between metabolic intensity, longevity, and actuarial aging using data from the literature and from field studies of exceptional size-dependent longevity.

MATERIALS AND METHODS

Published data on record longevities and body mass of marsupials have been logarithmically transformed and analyzed by least squares regression:

log life span = log a + b log body mass

Antilogarithmic forms of such equations are compared in terms of the coefficient *a*, which is characteristic of a phylogenetic group, and exponent *b*, which shows the scaling within the group. The exponents *b* that would be obtained via the reduced major axis regression model are obtained by dividing the least-squares *b* by the correlation coefficient *r*, and are noted below as *b*(rma).

Correlations of metabolic restriction and life span were taken from the literature and from a long-term banding study of the broad-tailed hummingbird (*Selasphorus platycercus*) at the Rocky Mountain Biological Laboratory (RMBL). Calder *et al.*, 1983, described this study site and the capture techniques.

RESULTS AND DISCUSSION

I. Interspecific longevity comparisons

A. Interclass comparisons. Lindstedt and Calder (1976) published the allometry of maximum lifespan (t,y) for birds (mol = body mass, kg.):

for captive birds [$r = 0.70$; b(rma) = 0.26]: $t = 28.3\ M^{0.19}$ (2)

for wild birds [$r = 0.78$; b(rma) = 0.27]: $t = 17.6\ M^{0.20}$ (3)

for wild birds, excluding Passeriformes: $t = 16.6\ M^{0.19}$ (4)

Compared with eutherian mammals (Eqn. 1), the birds' tissues and metabolism appear twice as durable. What about the other homeotherms?

for captive marsupials [$r = 0.51$; b(rma) = 0.37]: $t = 6.15\ M^{0.19}$ (5)

[$P < 0.001$; s.e.(b) = 0.051; s.e.(log a) = 0.0441; $n = 42$ species from Jones, 1982; body

masses from Strahan (1983) for Australian marsupials and from Nowak and Paradiso (1983) for New World species; the 95% confidence limits of a do not reach the eutherian intercept, Eqn. (4). Thus the marsupials are affected by size differences in parallel with birds and eutherians. Why the scaling factors should be so consistent between classes is a mystery. If the exponents were one fourth rather than one fifth (or if there is no statistically significant difference between these exponents), this could be rationalized as being proportional to metabolic turnover times (Lindstedt and Calder, 1981), as if a cumulative build-up of a metabolite or biochemical wear or disrepair ultimately limits life span. However, for a given size, marsupial longevity tends to be brief compared to eutherian mammals. On the other hand, a bird can outlive either type of mammal when size has been factored out. Equations 3 and 4 predict t_{max} = 5.68 to 6.00 years for a wild 3.5 g bird (compared to only 3.7 years for a 3.5 g mammal, even in the safety of captivity). Our record for the 3.5 g broad-tailed hummingbird is 8 years (Calder *et al.*, 1983), 41 and 33% longer than the predicted values.

B. Lifetime energy potential. There are many hypotheses for explaining why animals' life spans are finite, even in the absence of predation and disease. One is that there is some upper limit to the cumulative energy turnover per unit mass of tissue, the "metabolic life span." The total metabolic turnover in a lifetime is the product of the rate of energy turnover and the duration of life (t_{max}). Of course, wild animals (even captive ones) live at a variety of levels for various durations—waking, sitting, feeding, running, and so forth, but the standard or basal specific metabolism ($\dot{E}_s/M$, kJ per kg per unit time) gives at least a minimum value for this rate. The product $(\dot{E}_s/M) \times (t_{max})$ has been cited as an apparent constant within the eutheria in recent years by a number of writers (Stahl, 1962; Cutler, 1976, 1978; Lindstedt and Calder, 1981). This could be approached intraspecifically, by comparing cumulative metabolism with life spans of experimental individuals, or interspecifically, using the allometric cancellation technique of Stahl (1962). Within the eutheria the upper limit to cumulative lifetime energy turnover is seen interspecifically as a size-independent constant (Stahl, 1962; Cutler, 1976, 1978). In other words, the small mammal with intense specific metabolic rate burns out sooner whereas the large mammal lasts longer by metabolizing at a lower rate.

Let us first examine the concept in interspecific generalities: a class, supercohort, or order at a time. (See Table 1. The taxonomic level included in an allometric regression is a matter of empirical statistical physiology, rather than one of phylogenetic rigidity.)

The marsupials do not stretch their lives out by burning more conservatively, even though their standard metabolism tends to be only 70% as high as the eutheria (Dawson *et al.*, 1979). Do they exhibit relatively rapid aging that would make them of interest in

TABLE 1. LIFETIME STANDARD METABOLIC PRODUCT IN CAPTIVITY

Taxon	*Max. longevity* (years)	*Std. metabolism* MJ/(kg × yr)*	*Life energy* (MJ/kg)
Eutherian mammals	$11.6\ M^{0.20}$	$104.7\ M^{-0.25}$	$1215\ M^{-0.05}$
Marsupial mammals	$6.2\ M^{0.19}$	$73.7\ M^{-0.25}$	$456\ M^{-0.06}$
Nonpasserine birds	$28.3\ M^{0.19}$	$140.8\ M^{-0.27}$	$4008\ M^{-0.08}$

*converted from Calder (1974) and Dawson *et al.* (1979).

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experimental gerontology? Equation 5 is only a preliminary one. Although its significance is high, it incorporates far more variability (note r values). Perhaps this is a matter of limited sampling because relatively few marsupials have been maintained in zoos long enough to approach attainment of maximum life spans. In fact, six species ranging from 0.13 to 25.5 kg in size equal or exceed the eutherian predictions and four more attained 85% or more of the predicted eutherian "norms." Hence, one might expect to see longer records for many of the marsupials as more data are accumulated.

There are only three species of monotremes and they span only a 10-fold size range, so allometric regressions are inappropriate. However, their body masses can be used to predict "typical" maximum t and standard metabolism ($\dot{E}_s$) from eutherian equations for comparisons with observed values (t_{max} from Jones, 1982; $\dot{E}_s$ from Dawson *et al.*, 1979).

Ornithorhychus metabolizes at 66% of eutherian prediction and lives 139% as long for a lifetime E that is 92% of a 1.32 kg eutherian's. *Tachyglossus* metabolizes at 29% of eutherian, for up to 333% of predicted lifespan, 97% of the lifetime requirement of a 3.4 kg eutherian. *Zaglossus* burns at 26% and lives 157% as long for a total of 41% of the lifetime basal consumption of a 13.6 kg eutherian. At least for the two smaller monotreme species, a lower rate for a longer time gives a close quantitative approach to 1.0 to support the metabolic lifetime hypothesis.

The birds are clearly exceptional, living almost 2½ times as long and able to use over 3 times as much basal energy through that lifespan (Table 1). Hence the "secret" of avian longevity deserves further attention, but before the "secret" can be revealed, a stronger empirical information base is needed.

C. Life expectancy. A more realistic expression of lifetime energy turnover potential would be the product of realized lifespan and lifetime average metabolic rate. This would be approximated by the product of life expectancy and field metabolic rate based on observed time budget or actual determination of doubly-labelled water turnover. The product of life expectancy, $M^{1/3}$, and the average (field) metabolic rate per kg body mass, $M^{-1/3}$, (Calder, 1984a) is about twice the size-independent product of standard metabolism and maximum lifespan.

II. Intraspecific comparisons

A. Restricted energy intake. Now let us examine the "metabolic lifespan" concept intraspecifically. Life extension via restriction in energy turnover has been described, intraspecifically, in rodents. "It is apparent that underfeeding influences some basic biological phenomenon, which results in an increased lifespan. The biological mechanisms responsible for these effects are unknown." (Barrows and Roeder, 1977). It is well-known that restriction of energy intake is the most effective method for prolonging life and reducing risk of age-related diseases in laboratory *Rattus norvegicus* and *Mus musculus* (Ross, 1978; Masoro *et al.*, 1980; Leveille *et al.*, 1984).

B. Reduced energy turnover: Hibernation. During the hypothermic torpor of hibernation, the metabolic rates of mammals drop to 1/30 to 1/100 of normal basal metabolism (Hoffman, 1964). Lyman *et al.* (1981) found a strong correlation between the lifespan and the proportion of the life spent in hibernation by the Turkish hamster, *Mesocricetus brandti* (t_{ls} = age at death in days; $r = 0.522$, $P < 0.001$):

$$t_{hi} = 653.2 + (18.00 \times \text{percentage of life in hibernation}) \quad (6)$$

A ratio and proportion estimate from data in Lyman *et al.* (1981) suggests that the good hibernators live long enough to be able to consume 20% more basal energy than the poor hibernators. However, under the experimental conditions of 5½ months exposed to a temperature of 5°C, the poor hibernators would have to consume considerably more energy for the mean of 33 additional days of normal thermoregulation. The data necessary for reconstituting the entire energy budget were not contained in the report, but at first approximation it appears likely that the metabolic lifespan of (warm × fast) might equal (cool × slow). In other words, metabolic reduction should be inversely proportional to life span extension, and their product should approximate unity.

C. Reduced energy turnover: Hummingbird torpor? Cutler (1976) noted that in contrast to the well-known constant product of specific metabolic rate and maximum life span, that among homeotherms, those that undergo daily torpor, ". . . the bat and hummingbird do not fit this relation when the waking specific metabolic rate is used. Life spans of the bat and hummingbird appear too long in relation to their size." In other words, metabolic reduction should be inversely proportional to life span extension, and the product of these proportions should cancel at near unity.

Although Herreid's (1964) comparison of tropical (nontorpor) and temperate (torpor-utilizing) bats failed to confirm this, our hummingbirds seem to support Cutler. The 8-year maximum longevity of the broad-tailed hummingbird may be another case in support of the metabolic life span concept. Can the difference between observed (8 years) and predicted (5.7–6 years) life span of broad-tailed hummingbirds be related to the reduced metabolism of hypothermic torpor? Time-energy budgets with and without torpor have been compared in the Anna's hummingbird (*Calypte anna*). Estimates ($n = 4$; reviewed by Calder, 1974) assuming nocturnal torpor run 0.70 to 0.76 (mean 0.736) of the daily total if normal body temperature is maintained for all 24 hours. The Anna's hummingbird breeds in the winter in California and Arizona, facing cold stress similar to what is experienced by broad-tails at higher elevations. If the metabolic reductions estimated for Anna's are characteristic of other hummingbirds similarly exposed, a product of unity for the ratios of metabolic reduction and lifetime extension would support the metabolic lifespan concept:

$$\frac{\dot{E}\ \text{observed}}{\dot{E}\ \text{predicted}} \times \frac{t_{max}\ \text{observed}}{t_{max}\ \text{predicted}} = \frac{\text{lifetime } E}{\text{lifetime } E} \quad (7)$$

$$0.736 \times 1.41 = 1.04 \quad (8a)$$

$$0.736 \times 1.33 = 0.98 \quad (8b)$$

The total reproductive fitness of an animal is the product of rate and time, not only the average natality rate per breeding season, but its reproductive lifetime. The lifespan component ordinarily receives little attention because individual life histories are determined at great expense of time. However, it is this long-term component that would be especially important to a species facing an uncertain environment in which there may be bad years when the nectar supply and reproductive efforts fail, due to late snowstorms (Ehrlich

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et al., 1972), early decline of flowers, or excessive competition with migrant invaders (Calder, 1973). Reproductive success might then be defined by the ability to live long enough to span the gap between good years.

III. Other correlations with lifespan

Rose (1984) produced evidence that senescence can be postponed, using 15 generations of artificial selection for increased fertility at progressively later ages, in *Drosophila melanogaster*. This work supports the idea of an antagonistic pleiotropy mechanism for the evolution of senescence. However, in regard to pleiotropy, there is no information on many other aspects of the biology of the selected fruit flies. In particular, what happened to metabolic rates? If the selection was for flies with low metabolism, this could have been the basis of the delay of senescence.

It seems entirely possible that for any given body plan and size, there is a range of tradeoffs. If the product of longevity and specific metabolic rate were a constant, selection of reduced metabolic intensity may have bought greater longevity in exchange, so this might follow the metabolic lifespan hypothesis. For example, the product of t_{max} in eutherian mammals and basal metabolic rate appears to be a size-independent constant (Stahl, 1962; Cutler, 1976, 1978, 1983). The egg-laying monotremes, as noted above, have considerably lower metabolic rates and considerably longer lives. Turkish hamsters live predictably longer in proportion to the percentage of lifespan spent in hibernation, a period of greatly reduced metabolic rate.

Superoxide dismutase (SOD) serves to detoxify free radical oxide ($O_2^{-\bullet}$) generated in aerobic metabolism. If production of these free radicals is proportional to oxygen consumption rate per g of tissue, and the latter approaches a lifetime constant, then it is likely that free radical production is a constant for birds and similarly for mammals (Lindstedt, in press; Cutler, 1983).

Ono and Okada (1984) reported a good positive correlation ($r = 0.644$) between brain SOD-specific activity and maximum lifespans of 11 mammal species ranging in size from mouse to horse. No positive correlations were found for five other enzymes.

Life span or maximum longevity has been frequently correlated with other characteristics, such as body mass, brain mass, and spleen mass, for example. Correlation exercises are useful guides for our thinking, but they cannot prove causation. Lindstedt and Calder (1981) found an overwhelming similarity in the scaling of physiological time scales to body mass, from the brief cycle of a fast muscle twitch to the time required for an entire life cycle.

One could therefore argue that SOD activities and life spans are both functions of body size, and are only indirectly related through the metabolic lifespan. Being correlated with a common characteristic, body size, they would naturally correlate with each other.

For example, regressing the data of Ono and Okada (1984) on body mass (M, kg) for these species, there is an even better correlation ($r = 0.829$):

$$\text{SOD specific activity} = 12.84\ M^{0.07 \pm 0.015} \tag{9}$$

There is less variability in brain mass than in body mass, so a regression for SOD on brain mass would, no doubt, yield an even higher correlation coefficient (see Lindstedt and Calder, 1981). All of this does not disprove the explanation by Ono and Okada that life

span and SOD activity are directly related; it merely says that there are many ways of obtaining good correlations to raise further questions.

It is worth recalling that Emmett and Hochachka (1981) found good positive correlations ($r = 0.71$ to 0.80) for 3 glycolytic enzymes in muscle (including LDH used by Ono and Okada). However, the correlations were inversely related to size for three oxidative enzymes ($r = -0.61$ to -0.72).

IV. Hummingbird population biology: A natural aging model

Aging is so rarely observed in wild animals that it is usually assumed to be biologically insignificant (Botkin and Miller, 1974; Cutler, 1983). Nevertheless, it is a biological phenomenon that must be occurring, if only rarely, or perhaps it is more common than our techniques of study reveal. The allometry of age-dependent mortality has been calculated, but only from published values from captive mammals (Calder, 1982, 1983, 1984a; using data of Sacher, 1978). There appears to be no parallel information on birds (Lindstedt, in press). It is well-recognized that maximum longevity for birds is longer than for mammals (see above). Is this the result of a lower vulnerability throughout life (q_o of Sacher, 1977), perhaps conferred by higher body temperatures and greater resistance to bacterial infection? Or do birds age at a lower rate (α of Sacher, 1978)?

Other than making the point that aging must occur in birds, as in mammals (Botkin and Miller, 1974; Calder, 1983, 1984a) there has been little study of aging in wild birds, due mostly to difficulties in obtaining adequate data above the often swamping effect of age-independent factors such as predation and accident. Thus the allometry of avian life expectancy is not yet clear.

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On both of Botkin and Miller's (1974) criteria, length of time and numbers of birds necessary for proper age-dependency analysis, the population of broad-tailed hummingbirds studied at RMBL for 14 years (saturation banding since 1979) is ideally suited for the integrated study of longevity, survivorship, and the effect of metabolic reduction during torpor in enhancing these (Calder, 1984b; Wiens, 1984). The nectar-feeding habits of hummingbirds are easily transferred to artificial feeders. When the numbers and locations of feeders are controlled at a biological research station it is possible to attract essentially all of a local population for capture. Although much remains to be learned of the biology of this population, it is probably safe to claim that this is becoming one of the most extensively-studied populations of birds in North America. Pollination biology, foraging strategy, competition, territorial and nesting energetics and behavior, nesting success, longevity and population dynamics, and the physiology of torpor, cardiovascular, and osmoregulatory systems have all been studied in the same population by several biologists. Now we approach the point of being able to conceptualize functional relationships and better understand the integrated life history.

The progress and potential of this banding/recapture effort is seen in Figure 1, which summarizes 223 recaptures of adult female broad-tailed hummingbirds (solid curve). In the period 1973–1978, banding was incidental to other projects, and we (David Inouye, University of Maryland, Nickolas Waser, University of California, Riverside, and our assistants) averaged 11 adult female bandings and 9 recaptures. After the efforts were intensified in 1979 until most if not all of the population was banded (at the only three artificial feeder locations and in mist nets throughout the area), the annual average rose to 31 new female bandings (adults) and 33 females recaptured. Pooled recapture data from

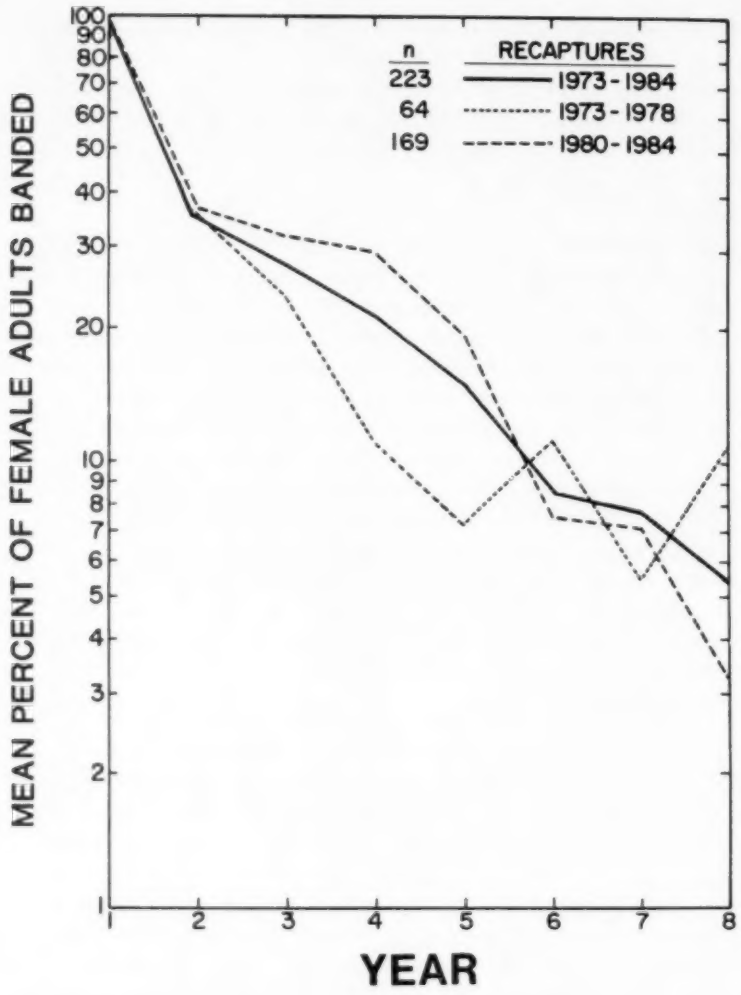

FIG. 1. Recaptures of female broad-tailed hummingbirds as mean percentage of number of birds originally banded as a function of years since banding as adults in year 1. Note that in the years since 1979, when efforts were intensified to band and recapture essentially all of the local population, that the curve (dashed) approaches the Gompertzian shape, permitting distinction between age-dependent and age-independent mortality. The initial steep decline from year 1 to year 2 probably represents transient birds that were not part of the local breeding population, and whose failure to be recaptured does not therefore represent actual mortality.

the first "incidental" period (Fig. 1.) show no clear pattern. However, pooled data from the 1980–1984 "intensive" period (----) suggest a Gompertzian shape or "type I" survivorship curve from which age-independent or initial vulnerability and age-dependent (actuarial aging rate) can begin to be distinguished.

Because these data are for adult females only, the initial steep decline from banding year 1 to year 2 (first recapture) must represent the fact that many bandings were of transient females, not part of the local breeding population. Extrapolation of the slopes between years 2 and 4 suggests that between 46% and 59% of the bands were placed on migrants (the species breeds northward through Wyoming and Utah) or post-reproductive wanderers (the species breeds from 1600 m upward; nesting can be completed earlier at the lower elevations). Such birds would be far less likely to be recaptured in subsequent years, thereby diluting the apparent survivorship. Thus the actual mortality may be considerably less than implied in the age-independent curve of Calder *et al.* (1983).

The returning females of years 2 to 4 show a mortality rate of about 0.106 years, which is only 36% of the initial mortality rate (q_o) predicted for a 3.5 g mammal (from eqn. 18 of Calder, 1983). From year 4 to 6, there was an approximately 27% annual increase in mortality, less than 10% of the actuarial aging rate (α) predicted for a 3.5 g mammal.

Miller and Gass (1985) reviewed the records and concluded that predation risk is not a significant factor in hummingbird mortality. This is another reason why it seems possible to see the effects of aging on this survivorship curve.

Thus, the superior longevity of birds among homeothermic vertebrates, and of hummingbirds among birds, the ability of hummingbirds to reduce metabolism in torpor, and their apparent relative invulnerability to predators, all combine to suggest that hummingbirds could be valuable models for field studies of longevity.

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GENETIC CONTROL OF ADULT LIFESPAN IN *DROSOPHILA MELANOGASTER*

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Abstract—Two temperature-sensitive X-linked adult lethal mutations were induced by ethyl methane sulfonate in *Drosophila melanogaster*. These mutations exhibited both a temperature-sensitive and effective lethal phase in adult flies. The existence of this new class of gene mutations provides experimental support for the hypothesis of H.J. Müller (1963, p. 235) that "development is a continuous process of which senility forms the last state." Furthermore, their existence demonstrates that the role of genes in controlling adult lifespan may be dissected by those methods that have been used to successfully probe the genetic control of preadult development.

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INTRODUCTION

A SATISFACTORY causal explanation of the mechanism for senescence in any organism remains unknown. However, numerous hypotheses have been posited in an attempt to provide theoretical constructs from which to initiate experimental approaches to this biological problem. H.J. Müller suggested that "development is a continuous process, of which senility forms the last state" (Müller, 1963, p. 235). Since development is synonymous de facto with differential gene activity, it thereby follows that if this suggestion is valid, it should be possible to dissect the genetic control of adult lifespan, and of senescence or adult development, by application of those genetic techniques that have been successfully used to probe and thereby gain understanding of preadult development.

The most direct approach towards demonstrating that senescence results from differential gene activity in adult forms, and is therefore but the final stage of a continuous process of development, is to select for mutations of genes that are activated only in adult organisms. The efficacy of this selection can, in theory, be demonstrated by isolating adult phase-specific conditional lethal mutations (e.g. temperature-sensitive) (Suzuki *et al.* 1967; Grigliatti and Suzuki, 1970; Grigliatti and Suzuki, 1971; Grigliatti *et al.*, 1972; Fullilove and Woodruff, 1974) with *both* a temperature-sensitive period and effective lethal phase in the adult organism.

This study reports the induction and selection of two temperature-sensitive X-linked (X-chromosome) adult lethal mutations in *Drosophila melanogaster* that exhibit both the temperature-sensitive and effective lethal phases only in adult flies.

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MATERIALS AND METHODS

Inbred Oregon R (*OrRB*, wild-type) and Basc (Müller-5: In (1) $pc^{sl}L pc^{sR}$ + 5, $sc^{8}ec^{sl}w^{a}B$) strains of *Drosophila melanogaster* were used in these studies. Stocks were originally obtained from the laboratory of Arnold M. Clark, The University of Delaware, Newark, Delaware. Fly stocks were maintained at 25°C, 65% RH, 12 h: 12 h light-dark cycle in 25 × 93 mm foam-stoppered glass tubes containing 8 ml of standard cornmeal–molasses–agar medium poured on a slant.

The method used to induce X-linked ethylmethane sulfonate (EMS) induced mutations in *Drosophila* has been previously described (Pelecanos and Alderson, 1964). We expanded upon this basic scheme to select for X-linked temperature-sensitive adult lethal mutations by utilizing the Basc (Müller-5) method (Figure 1). Newly emerged *OrRB* males were collected and treated overnight by maintaining flies in cotton-stoppered 22 × 150 mm tubes containing 2 cm of tightly-packed Kleenex saturated with 15 m*M* EMS dissolved in 10% sucrose. Treated males were mated for 24 h to virgin Basc females. Mated females were then transferred to tubes containing standard cornmeal–molasses–agar medium. F_1 females tentatively heterozygous for induced mutations were mated to their brothers, and F_2 males (usually 2–10 from the initial 24 h period of oviposition), which may be hemizygous for life-shortening mutations, were tested for decreased longevity by determining their survivorship on white cornmeal medium (Strehler, 1961) at 30°C in 16 × 100 mm foam-stoppered tubes. Those lines in which 50% or more of the flies died before 21 days of age at 30°C were tentatively characterized as bearing putative X-linked life-shortening mutations. All of these lines were then retested by rearing flies at 25°C and determining the life-span of flies maintained on white cornmeal medium at varying temperatures.

Fifty flies (ten flies per tube) were used to generate each survivorship curve. The number of surviving flies were recorded daily and survivors were transferred to fresh medium at two-day intervals. All experiments were carried out at least two times.

RESULTS

In the initial study, 144 EMS-treated X-chromosomes were tested for the presence of life-shortening mutations utilizing this selection scheme. Fifty-four female flies were sterile, forty flies carried X-linked pre-adult lethal mutations, and eight female flies were isolated, which produced sons exhibiting a reduced lifespan phenotype. Thus, 1–2% of all EMS-treated X-chromosomes carried lifespan-shortening mutations, a frequency similar to that previously reported by others (Baird and Clark, 1971; Gould and Clark, 1977).

1. Expose OrRB males overnight to EMS ($\overset{*}{X}$/Y)
2. P $\overset{*}{X}$/Y x Basc/Basc

   F_1 Basc/Y x $\overset{*}{X}$/Basc

   F_2 $\overset{*}{X}$/Y, Basc/Y, $\overset{*}{X}$/Basc, Basc/Basc
3. Select for decreased longevity of mutant males at 30°C.
4. Retest putative life-shortening mutations.
5. Test mutants for longevity at 25°C.

FIG. 1. Protocol used to select for EMS-induced X-linked conditional adult lethal mutations in *Drosophila melanogaster*.

The survivorship curves determined at 30°C for male flies from two mutant lines, designated 1001 and 1002, as well as that for *OrRB* control males are shown in Figure 2. It is clear from this figure that the adult lifespan of males from these two lines, when determined at 30°C, are virtually identical, and markedly shortened, relative to that of wild-type male flies.

One of these mutations (1001) was found to be temperature-sensitive upon further testing. The lifespan characteristics of mutant males bearing this mutation, as well as that for line 1002 and for control flies is shown in Figure 3. The lifespan of flies reared at 25°C and determined at 30°C confirms the findings in Figure 2 (Figure 3, top panel). When flies from both mutant lines, as well as control flies, were reared at 25°C and the duration of their adult lifespan determined at 25°C, there was an increase in life expectancy for all flies, as expected for poikilothermic insects (Loeb and Northrop, 1917; Alpatov and Pearl, 1929; Clark and Maynard-Smith, 1961; Strehler, 1961; Hollingsworth, 1969). However, the effect of temperature upon the increase in lifespan was greater for flies from line 1001 than for flies from line 1002 (Figure 3, middle panel). This difference in lifespan

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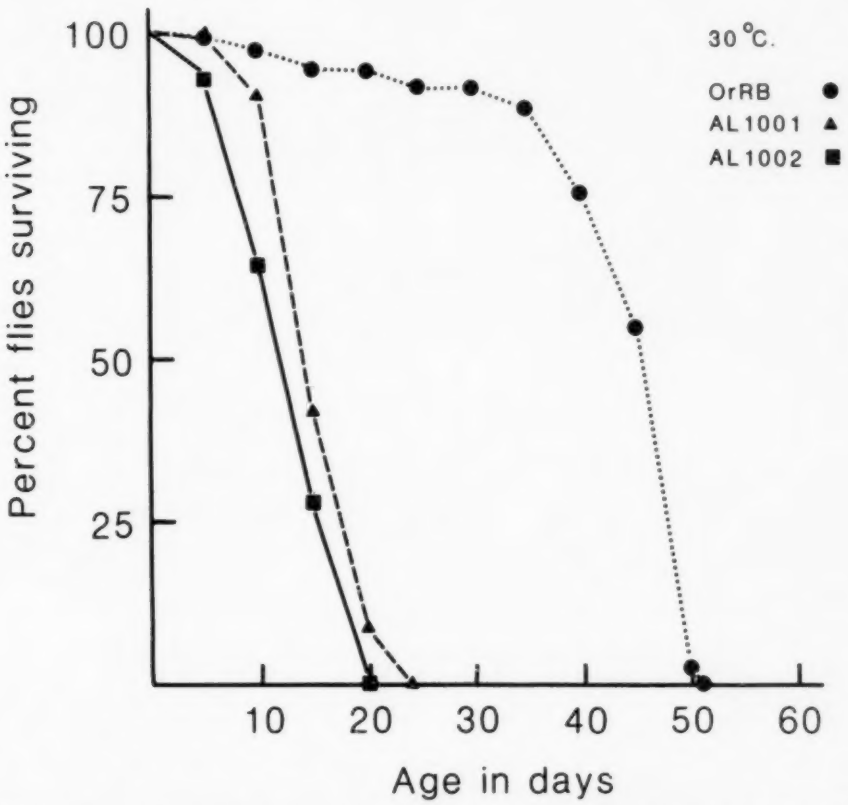

FIG. 2. Adult lifespan of *Drosophila melanogaster* males reared at 25°C and maintained at 30°C for duration of adult life.

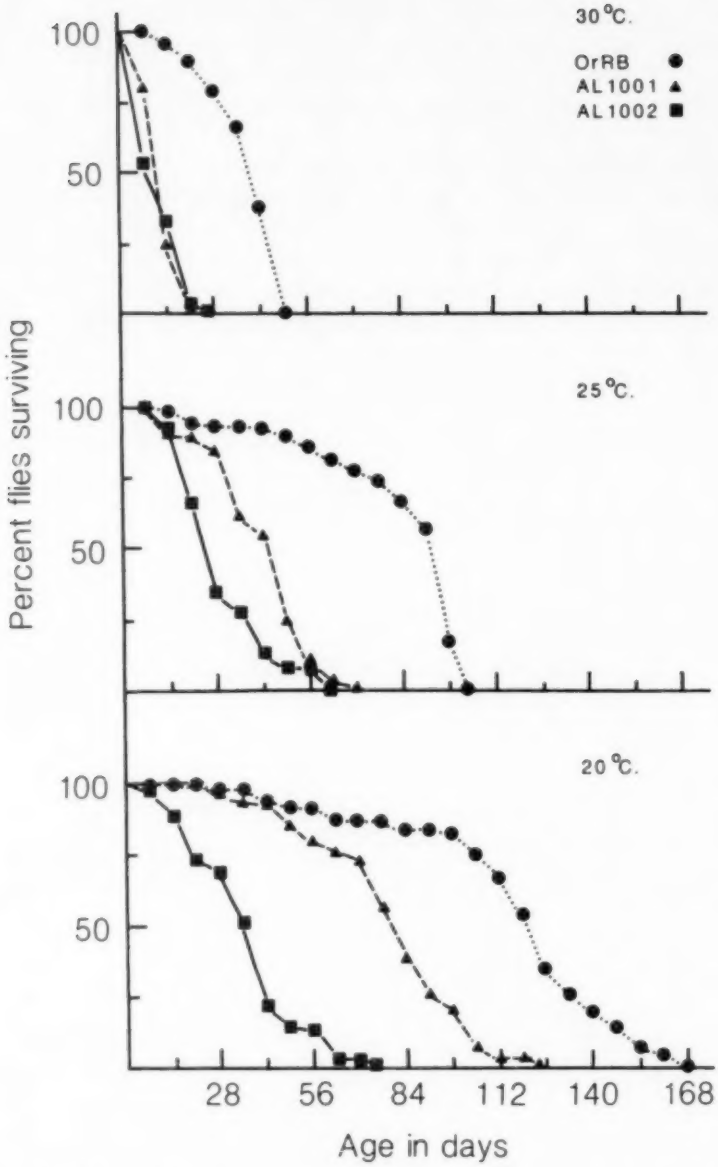

FIG. 3. Survivorship of *Drosophila melanogaster* males reared at 25°C and maintained at 30°C, 25°C, or 20°C for the duration of their adult lifespans.

in response to a decreasing temperature became even more apparent when flies were reared at 25°C, and their adult lifespan determined at 20°C (Figure 3, bottom panel).

These data show that flies from line 1001 bear an X-linked conditional adult lethal mutation. Additional support for this conclusion was obtained from studies that showed that

the adult lifespan of 1001 males was not affected by the temperature at which the flies were reared (20°, 25°, or 30°C, data not shown), thus reducing (but not eliminating), the possibility that this mutation is a conditional preadult lethal mutation with the effective lethal phase in the adult organism.

Yet another mutant line, designated 1003, was induced by ethylmethane sulfonate as described and isolated in a subsequent study. Male and female flies from this line, as well as control flies, were produced at 25°C, and the lifespan of the adults determined at 30°C, 25°C, and 11°C. The results are presented in Figure 4. It is clear from this study that there is little difference in the lifespan of males and females of strain *OrRB* of *Drosophila.* Mu-

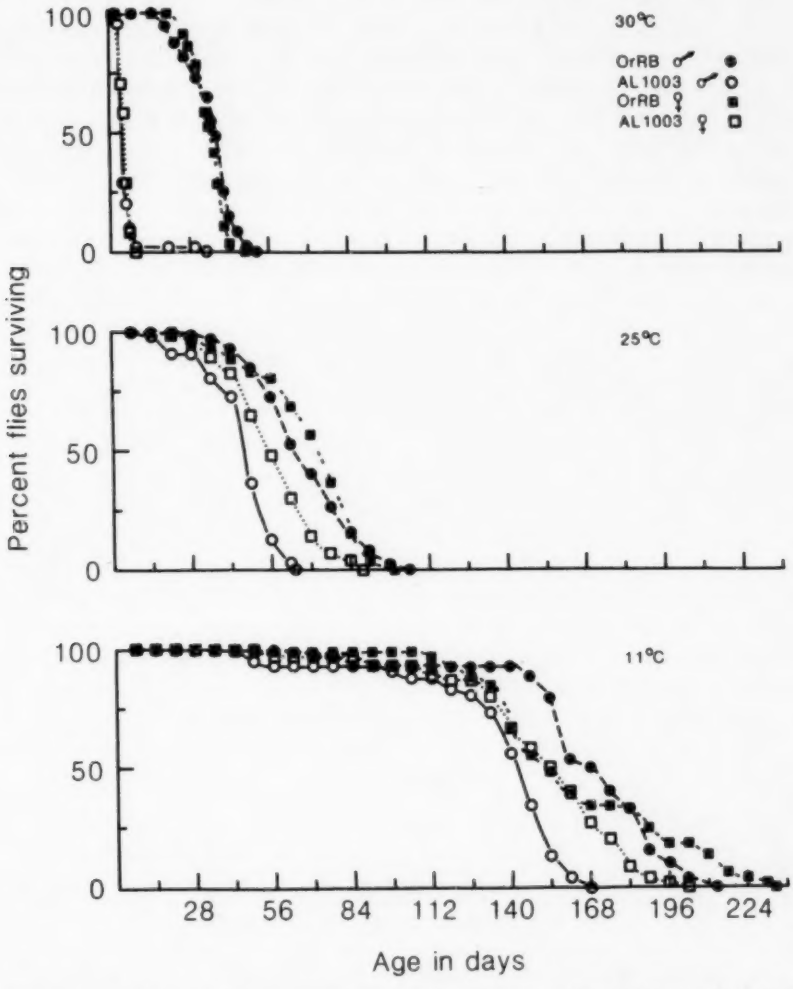

FIG. 4. Adult lifespan of *Drosophila melanogaster* male and female mutant flies (1003) reared at 25°C and maintained at 30°C, 25°C, or 11°C for the duration of their adult lifespan. Surviving flies maintained at 11°C were recorded and transferred to fresh medium at weekly intervals.

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tant male and female flies exhibit a marked decrease in life expectancy at 30°C (Figure 4, top panel). However, lowering the maintenance temperature results in a much greater proportional increase in the lifespan of mutant flies, such that there is little or no significant difference in the life expectancy between male and female mutant or control flies maintained at 11°C (Figure 4, bottom panel).

DISCUSSION

Lethal factor analysis has been utilized extensively in order to elucidate the genetic control of preadult development, especially in insects (Hadorn, 1961). This approach has also been previously exploited in an attempt to understand the role of genetic factors in controlling aging and longevity. Thus, so-called adult lethal mutations (Medawar, 1957) have been induced and isolated in both the wasp, *Habrobracon* (Baird and Clark, 1971) and in *Drosophila* (Gould and Clark, 1977). Furthermore, an attempt has been made to localize the site of action of such mutations in *Drosophila* by use of mosaics (Gould and Clark, 1983). However, although it is of obvious interest to localize the site of action of any mutation that alters adult lifespan, previous studies are subject to the patently valid criticism that mutations that shorten adult lifespan may actually affect genes that are active during preadult development. In this instance, the life-shortening effect would be the *consequence* of preadult gene action, rather than the result of gene activity in adult forms. Therefore, an acceptable test of Müller's hypothesis requires the demonstration of differential gene action in adult organisms. Such demonstration concomitantly signals that the process of senescence can, in fact, be experimentally examined by those same genetic approaches that have been applied in studying preadult development. The successful isolation of the two X-linked conditional life shortening mutations reported herein appears to offer strong support for Müller's hypothesis.

We do not as yet know what process (or processes) the wild-type alleles of these mutant genes normally control. However, the successful selection for a mutation of gene(s) with both the temperature-sensitive period and effective lethal period in the adult organism provides strong evidence that genes act in a differential fashion in adult organisms and offers support for the hypothesis that senescence is the final stage of development. Furthermore, the results reported herein not only support the validity of Müller's hypothesis, but also provide experimental material for a noval approach to an understanding of the duration of adult lifespan, and possibly aging, by methods of lethal factor analysis, which have been so successfully exploited in providing the basis for much of our understanding of many aspects of the genetic control of preadult development.

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ASSOCIATION OF LIPID PEROXIDATION DURING LUTEAL REGRESSION IN THE RAT AND NATURAL AGING IN THE ROTIFER

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Abstract—Lipid peroxides (LP) were measured in whole homogenates and membrane fractions from luteinized rat ovaries and in homogenates of aging rotifers. There was a significant increase in the accumulation of LP as plasma progesterone levels declined during luteal regression. Lipid peroxide levels also increased with age in rotifers. This study indicates that lipid peroxide accumulation is associated with cellular breakdown and suggests that the rat corpus luteum may be used as a mammalian aging model.

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INTRODUCTION

A NUMBER of different models have been used to study the aging process. Invertebrates such as *Drosophila* (Pittendrigh and Minis, 1972), nematodes (Zuckerman and Geist, 1983) and rotifers (Sawada and Enesco, 1984a,b) are frequently employed, and they possess many advantages, including a short lifespan. Longer lifespan and increased complexity make studies of vertebrates more difficult. However, various models have been used successfully to gain information about aging phenomena in vertebrates. These include cultures of human fibroblasts (Hayflick, 1980) and the use of placenta (Rosso, 1976; Fox and Faulk, 1981). Aging systems in mammalian species have also been examined for structural and functional alterations in situ. A number of limitations arise from these studies and additional systems would improve our understanding of the aging process. The purpose of this investigation was to examine the regressing corpus luteum (CL) as a possible aging model.

The CL develops after ovulation and secretes progesterone for the support of pregnancy. It is a temporary endocrine tissue that undergoes regression if conception does not occur. The first sign of regression is a fall in blood progesterone concentration, which is followed by morphological alterations as the luteal cells break down (Pharriss *et al.*, 1972). There are several advantages in using this tissue for aging studies. For example, it has a relatively short life span of approximately 2 weeks; degeneration of the CL can be induced experimentally by the administration of prostaglandin $F_2\alpha$ ($PGF_2\alpha$), the natural luteolytic agent; and functional changes can be easily assessed by measuring progesterone levels in the blood.

A comparative study was undertaken using the rotifer, *Asplanchna brightwelli*. This

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species has a short life span of about 5 days. Although it is an invertebrate, some interesting parallels with vertebrates have emerged from previous studies. As in rats and mice, dietary restriction significantly increases the life span of rotifers (Sawada and Enesco, 1984b). Furthermore, vitamin E, a free radical scavenger, has been found to increase the maximum longevity of mice (Ledvina and Hodanova, 1980), extend lifespan of cultured human fibroblasts (Packer and Smith, 1977), and increase mean life span in the rotifer as well (Sawada and Enesco, 1984a).

The auto-oxidation of polyunsaturated fatty acids increases during the aging process (Logani and Davies, 1980) and gives rise to lipid peroxides (Harman, 1981; Tappel, 1980). In the present study, lipid peroxides (LP) were measured in the regressing rat CL and in aging rotifers to determine if these models manifest similar chemical changes as observed in other aging systems.

METHODS AND PROCEDURE

Animals

Immature 3½-week-old female Wistar rats were superovulated by SC injection of 50 IU of pregnant mare serum gonadotropin (PMSG, Equinex, Ayerst Laboratories, Montreal, PQ) followed 65 hours later (Day 0) by 50 IU of human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, MO). This treatment provides an ovary that is heavily luteinized and is used frequently to study corpus luteum function in the rat (Armstrong and Greep, 1962; Bramley and Ryan, 1978). On Day 8 one group was injected SC with 500 μg of $PGF_2\alpha$ to induce luteal regression, while a second group was injected with 50 μl of saline (control). A third group of rats was not superovulated (untreated controls) to determine whether the PMSG-hCG treatment produces LP. Because spontaneous regression starts approximately 2 weeks after hCG injection, a fourth group was examined 17 days following hCG to determine the spontaneous changes associated with the breakdown process. Each group contained eight rats. Prior to killing the rats by cervical dislocation, blood samples were collected and plasma progesterone concentrations were measured by radioimmunoassay (Carlson and Gole, 1978) to assess luteal function.

Adult rotifers, *Asplanchna brightwelli* clone 4B61 (A gift from Dr. H.E. Enesco, Concordia University, Montreal, PQ), were placed 15 to a well into each well of a six-well tissue culture dish (Falcon No. 3046, Becton Dickinson & Co., Oxnard, CA) along with 8 ml of paramecia medium per well. Ten newborn rotifers were then placed into separate wells where they were maintained at 19°C throughout the experiment. Twice daily, 4 ml of the paramecia medium was replaced with fresh medium to maintain a constant food supply as well as to remove offspring that are normally born after 2½ days of age.

Paramecia medium

Paramecium caudatum, the food source of the rotifers, were cultured in a Cerophyll infusion medium. The method outlined is a modification of procedures described by Enesco and Verdone-Smith (1980) and Sawada (1983). A 4-litre flask containing 1900 ml of distilled water, 0.14 g $CaCO_3$ and 14.0 ml of 0.25 *M* phosphate buffer (pH 6.8) was heated to boiling. A 1.4 g sample of Cerophyll (Ward's Natural Science Est., Rochester, NY) suspended in 56 ml of distilled water was added to the mixture followed by a 30 ml rinse of distilled water. After boiling for 20 minutes, the solution was cooled in an ice bath and filtered through Whatman No. 1 qualitative filter paper. The solution was autoclaved for 20 minutes, cooled to room temperature, and innoculated with *E.coli* K12 (Ward's Natural Science Est.) for 24 hours at 37°C. A 100 ml sample of *Paramecium caudatum* (Boreal Laboratories Ltd., Mississauga, Ont.) was added to the solution and left at room temperature for 72 hours before use. *E.coli* was present to act as a food source for the paramecia as well as to prevent additional bacterial contamination.

LP measurements

LP levels in homogenates and membrane preparations from rat ovaries and in homogenates of rotifers were measured by the thiobarbituric acid (TBA) procedure described by Uchiyama and Mihara (1978). It was necessary to modify this procedure to measure LP in rotifers because it was impossible to obtain accurate sample weights because of the small size of this aquatic organism. Instead, rotifers were counted and greater numbers of younger animals were used to compensate for the increase in size with age (Sawada and Enesco, 1984a). Exactly 250 rotifers were used for the 6 hour point, 210 for Day 1, 180 for Day 2, 150 for Day 3, and 110 for Day 5. Each sample was brought to a final volume of 250 μl with distilled water, homogenized; 1.5 ml of 1% orthophosphoric

acid (pH 2.0), and 0.5 ml of 0.6% TBA were added. The mixture was boiled for 45 min, and cooled. Two ml of butanol-1 was added and the sample was mixed. The butanol layer was separated by centrifugation and its TBA value was measured as the difference of OD at 535 and 520 nm.

Membrane preparation

Cell membrane preparations were obtained using a modified dextran polyethylene glycol two-phase system (Brunette and Till, 1971). For each preparation eight pairs of ovaries were excised, combined, and homogenized at 4°C in 50 m*M* phosphate buffer (pH 7.8) with 12 strokes of a motor-driven Teflon-glass homogenizer. The homogenate was filtered through a 75 μm nitex filter and centrifuged for 20 min at 2000 *g* (4°C, Sorvall RC2-B, Norwalk, CT). The pellet was resuspended in 10 ml of 30% polyethylene glycol and mixed after the addition of 12 ml of dextran. The mixture was centrifuged for 15 min at 12,000 *g*, and the membrane located at the interface was removed and diluted with approximately four times its volume with 50 m*M* phosphate buffer. The solution was mixed and centrifuged at 10,000 *g* for 15 min to collect the membrane fraction.

Marker enzymes

Membrane marker enzymes were assayed to determine membrane origin and enrichment and these have been reported previously in our studies (Carlson *et al.*, 1984). The plasma membrane marker was 5'-Nucleotidase (Currie, 1980). NADH Cytochrome C reductase (Sottocasa *et al.*, 1967) served as the endoplasmic reticulum marker and succinate dehydrogenase activity (Pennington, 1961) was used as the mitochondrial marker. Protein content was measured by the Bradford method (1976), using bovine serum albumin as a standard.

Statistical analysis

Statistical analyses were performed with a statistical package that was developed in our laboratory based on procedures as indicated by Sokal and Rohlf (1981) and Brunning and Kintz (1977). A one-way analysis of variance (ANOVA) was performed on all data followed by a post hoc Tukey or Duncan's Multiple Range Test, depending on whether a single critical difference or a stairstep approach for making multiple comparisons was needed. Unequal sample size was also taken into account by using the harmonic mean of the numbers where applicable. Comparison of slopes was also analyzed for statistical significance.

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RESULTS

Table 1 shows the results of the marker enzyme assays that were used to determine the source and relative amounts of membrane in the preparation. The plasma membrane marker revealed an enrichment of 7.8 fold and 9.2 fold for the control and the $PGF_2\alpha$ treated group, respectively. However, the endoplasmic reticulum marker indicated that membranes from this organelle were also present in both groups, but there appeared to be little contribution from the mitochondria. Thus, the membrane procedure results in a preparation primarily enriched in plasma membrane.

TABLE 1. ENRICHMENT OF ENZYME ACTIVITY IN MEMBRANE PREPARATIONS (n = 3 ± S.D.)

	5'-Nucleotidase	*NADH Cytochrome C reductase*	*Succinate dehydrogenase*
Control	7.8 ± 2.3	4.1 ± 1.1	0.5 ± 0.25
$PGF_2\alpha$	9.2 ± 1.7	4.0 ± 0.8	0.4 ± 0.15

Each *n* represents a membrane preparation containing eight pairs of ovaries.

Enrichment was determined by dividing the specific activity of the membrane preparation by the specific activity of the homogenate.

Figure 1 shows the decrease in the plasma progesterone levels following a single SC injection of 500 μg of $PGF_2\alpha$. One-way ANOVA followed by Duncan's Multiple-Range Test indicated that the drop in plasma progesterone was significant ($\alpha = 0.05$) starting with the sample collected 0.5 hours after treatment.

The results of measuring LP in homogenates of ovaries removed from the controls and the $PGF_2\alpha$-treated rats appear in Figure 2. This experiment included two groups of controls, only one of which received the superovulation treatment. The second was untreated with gonadotropic hormones. A progressive increase in relative absorbance, indicative of an elevation in LP, appeared in samples prepared from regressing luteal tissue in the $PGF_2\alpha$-treated group, which was not observed in either control group. One-way ANOVA and subsequent post hoc Tukey Test indicated a significant difference between the regressing group and either control group from Day 1 onward after $PGF_2\alpha$ treatment. Analysis of regression lines showed that there was also a significant difference in the slope between samples from the $PGF_2\alpha$ treated rats and the two controls. In addition, results of LP levels in luteal tissue from superovulated rats undergoing spontaneous regression appear in Table 2. One-way ANOVA indicated a significant increase ($P < 0.005$) between the TBA values obtained from the regressing and the untreated controls.

Membranes are a potential source of LP substrates. Analysis of the levels of these products in the membrane fraction also reveals a significantly greater amount from Day 1 onward in preparations from $PGF_2\alpha$-treated rats (Figure 3).

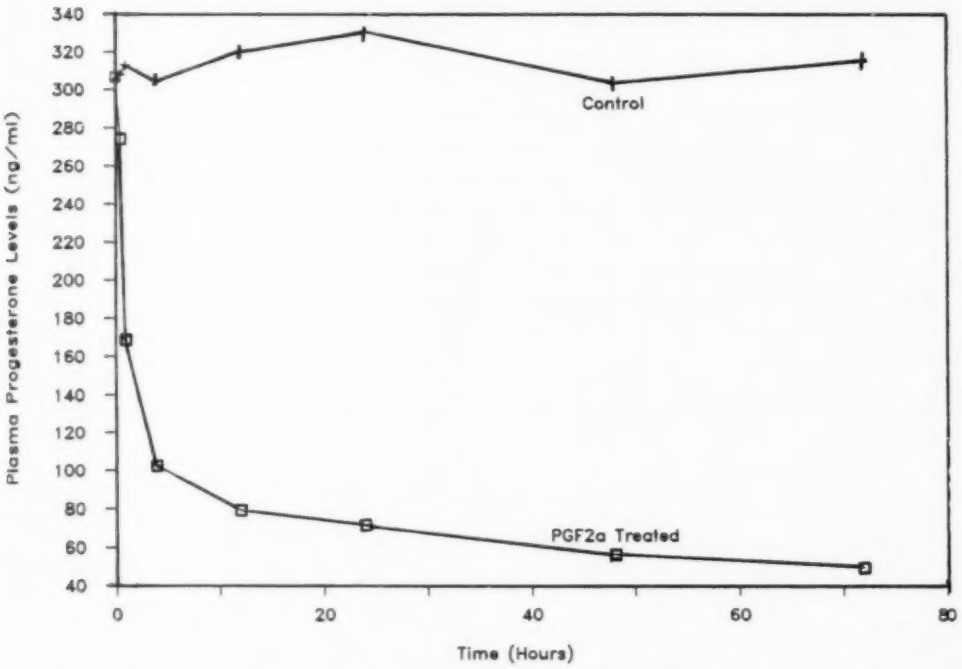

FIG. 1. Plasma progesterone levels in $PGF_2\alpha$ (500 μg, SC) treated and control rats. A significant ($\alpha = 0.05$) decline in progesterone levels, indicating a loss in endocrine function, began 30 min. following treatment.

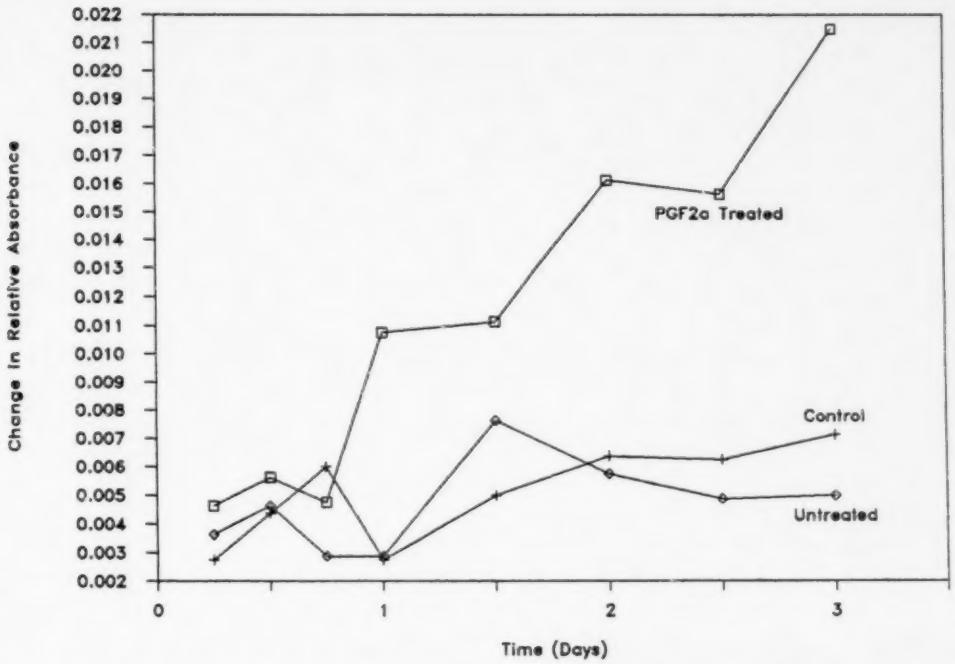

FIG. 2. LP alterations in ovarian homogenates from $PGF_2\alpha$ (500 μg, SC) treated and control (superovulated and untreated) rats. A significant increase in LP from Day 1 onward appears following $PGF_2\alpha$ injection.

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An age-related LP increase was also seen in the rotifer (Figure 4). One-way ANOVA followed by the Duncan's Multiple-Range Test showed that LP in 0.25-day-old rotifers were significantly lower than 2, 3, or 5-day-old rotifers ($\alpha = 0.05$) and that 1-day-old rotifers were significantly lower than 5-day-old rotifers ($\alpha = 0.05$).

DISCUSSION

Cellular degeneration and dysfunction occur during the aging process. These alterations appear in all systems, including the reproductive system. In female rats age-related changes include an imbalance in hormones secreted by the hypothalamic-pituitary-ovarian axis (Huang and Meites, 1975) as well as structural degeneration of oocytes (Peluso *et al.*,

TABLE 2. CHANGE IN RELATIVE ABSORBANCE (535–520 nm) BETWEEN OVARIAN HOMOGENATES ($n = 8 \pm$ S.D.)

	*Spontaneous regression**	*Untreated control***
OD difference	0.021 ± 0.009*	0.005 ± 0.003

*Significantly larger than untreated control ($P < 0.005$).
*17 days after hCG.
**not superovulated

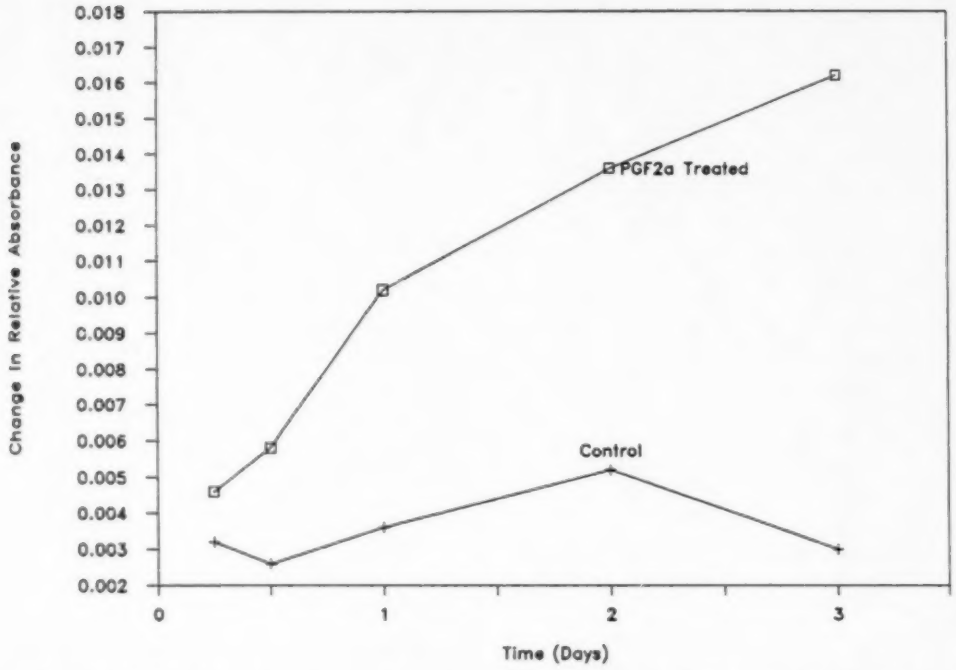

FIG. 3. LP alterations in membranes prepared from luteinized rat ovaries of $PGF_2\alpha$ (500 μg, SC) treated and control rats. A significant increase in LP from Day 1 onward appears following $PGF_2\alpha$ injection.

1980). As a result, reproductive cycles become more irregular, fertility declines, and ovarian activity ceases. In humans similar alterations in ovarian function occur at menopause.

There are a number of similarities between the aging process and regression of the CL at the end of the reproductive cycle. Functional and structural changes appear during luteal regression, as this tissue loses its ability to secrete progesterone. Ultrastructure studies indicate that the loss in hormone production is accompanied by cellular breakdown (Koering *et al.*, 1973). The present investigation was designed to compare lipid peroxide changes in the aging rotifer and regressing luteal tissue, because the former has been used previously for studying the aging process (Sawada and Enesco, 1984a). Comparable biochemical changes during cellular breakdown between these two systems would support further experiments with rat CL, which may also provide information about aging in a mammalian system.

In the present study, levels of lipid peroxides were found to increase in regressing luteal tissue and aging rotifers. Although the magnitude of the increase appears small, it is statistically significant in both systems and similar to the relative increase of these products observed in aging brain (Yoshikawa and Hirai, 1967), liver, kidney, and testis of the rat (Uchiyama and Mihara, 1978), as well as aging insects (Sohal *et al.*, 1981; McArthur and Sohal, 1982). In the present experiment, the elevation of these products in the rat ovary appears to be specific for samples prepared from regressing luteal tissue, because there was no apparent rise in the preparations from control rats untreated with gonadotropic

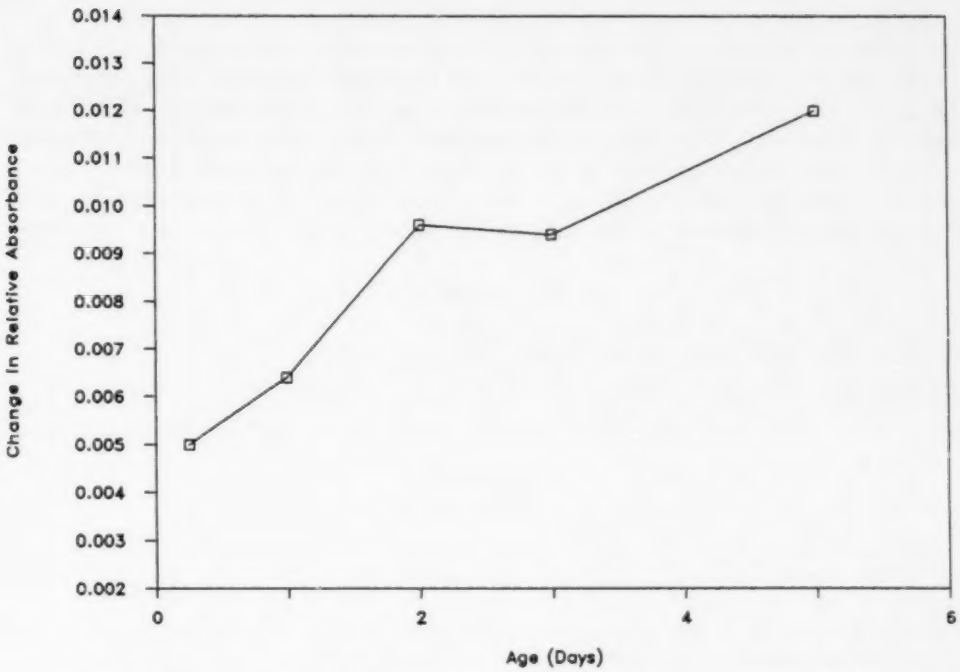

FIG. 4. LP alterations in aging rotifers. A significant increase in LP values appeared with age.

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hormones nor was it observed in samples from animals with functioning (i.e., nonregressing) luteal tissues. However, both regressing groups (spontaneous and $PGF_2\alpha$-treated) did manifest a parallel increase in the accumulation of LP. These results indicate that there may be similar chemical changes occurring during the degenerative process in aging rotifers and regressing luteal tissue.

The decline in progesterone secretion occurred a number of hours prior to the detection of an increase in LP in the present study. This indicates that there is a considerable gap between the first sign in the loss of function and an elevation in LP. Presumably this represents the time needed for various chemical changes to occur resulting in the appearance of cellular degeneration. A comparable delay has been reported in morphological studies. For example, Koering *et al.* (1973) report that during luteal regression, progesterone levels in the blood fall before the first signs of histological changes. Although the specific cause of these changes is unknown, there is evidence that it may involve the plasma membranes. Alterations in cellular membranes are a common feature of cellular breakdown and a number of examples have been documented by light and electron microscopy. In addition, fluorescence polarization studies indicate that there are also changes in membrane fluidity characteristics. For example, Rivnay *et al.* (1979) reported an increase in membrane viscosity in aging lymphocytes and spleen cells. A pronounced decrease in plasma membrane fluidity also appears during CL regression in the rat (Riley and Carlson, 1985).

As a result of the increase in lipid peroxides during luteal regression in rats and aging in rotifers, it seems possible that these products are related to the breakdown process. Loss

in cellular function may result from membrane alterations. Hochstein and Jain (1981) reported that lipid peroxides induce protein polymerization and membrane rigidification in human erythrocytes as they undergo changes in mechanical properties with age. Various intracellular sites can also be considered because these products alter the activities of some enzymes (Koster and Slee, 1980), which can result in disruption of various biochemical pathways. However, it should be noted that the lipid peroxide increase in the present study appeared after the initial decrease in progesterone secretion, which may mean that these products are not responsible for initiating the functional changes associated with regression.

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ASPARTATE METABOLISM IN RATS DURING AGING

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Abstract—(*U*-^{14}C)*L*-aspartate uptake and its conversion to CO_2, glutamate, glutamine, and GABA was studied in the parietal cortical slices from rats of various ages. Overall aspartate metabolism was enhanced during the aging period (55 and 80 weeks of age) as compared to the growing period (5 and 10 weeks of age).

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INTRODUCTION

ASPARTATE, GLUTAMATE, glutamine and γ-aminobutyrate (GABA) have closely related metabolic pathways. It has been generally accepted that aspartate and glutamate are excitatory neurotransmitters (Johnson, 1978), whereas GABA is thought to have an inhibitory action (Obata *et al.*, 1967). In the past few years, many studies suggest that there are neurotransmitter changes in the central nervous system with age. Studies on neurotransmitter changes might be important in order to clarify the mechanism of age-related physiological alterations such as motor dysfunction and declining mental activity. However, the changes in neurotransmitter concentrations alone give little information regarding the dynamics of metabolism. Smith *et al.* (1980), on the other hand, have demonstrated a widespread decrease of cerebral glucose utilization, significantly so in the parietal lobe of aging rats. Their observations indicate that the parietal lobe may be an appropriate region to study these age-related chemical changes. This investigation was performed to determine alterations in aspartate metabolism in the parietal cortex of rats with advancing age.

MATERIALS AND METHODS

Animals and slice preparation

Male Wistar rats were fed on Oriental Solid Food (Oriental, Itobashi, Tokyo) and given water ad libitum under specific pathogen-free conditions. The animals used were 5 and 10 weeks of age (growing period), and 55 and 80 weeks of age (aging period). They were killed by decapitation during fasting and the brains were removed immediately. After the meninges and vessels were removed and cooled in Gey's balanced salt solution (Gibco, Lawrence, MA), 0.5 mm brain slices were prepared from the parietal lobe with a Stadie-Riggs type slicer.

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In vitro assay

Brain slices (100 mg) were preincubated for 10 min in cooled Gey's balanced salt solution. Subsequently, the slices were placed in 3.0 ml O_2 saturated Gey's balanced salt solution (pH 7.4) in which 2 m*M* *L*-aspartate was added together with 0.5 μCi (U-^{14}C)*L*-aspartate (Amersham, Arlington Heights, IL). Tissues were incubated with mechanical shaking at 37°C for 45 min under O_2-saturated atmosphere, and incubation was terminated by the addition of 0.5 ml of 2 N HCl. After shaking, CO_2 liberated during incubation was trapped for 1 hour in Whatman No. 3 MM filter paper (Whatman, Maidstone, Kent, UK) (1.7 × 0.5 cm) immersed in 0.3 ml of 25% β-phenylethylamine-methanol (v/v). The slices, rinsed 3 times in physiological saline, were homogenized in 15-fold 75% ethanol twice. The homogenates were centrifuged at 4°C for 15 min at 3000 rpm. The supernatants were evaporated under an air stream. The extracts thus obtained were dissolved in distilled water and mixed vigorously with 3 ml of chloroform. An appropriate amount of the water phase was applied on a cellulose thin-layer chromatogram, which was run in two-dimensions, first with phenol-water (75 : 25, v/v) and then *n*-butanol-acetate-water (60 : 15 : 25, v/v). The chromatogram was sprayed with 0.15% ninhydrin-ethanol. When dry, it was heated in an oven at 75°C for 1 hr. Aspartate and its metabolites (glutamate, glutamine, and GABA), identified by superimposed amino acid standards, were cut from the chromatogram and placed in 0.5 ml of distilled water in a vial and shaken at 56°C for 1 hr. Thereafter, 5 ml of Intsa Gel (Packard, Downers Grove, IL) and one drop of acetic acid were added to the vial, and the radioactivity was measured with a Packard liquid scintillation spectrometer.

RESULTS

The amounts of radioactivity recovered from aspartate and its metabolites are shown in Table 1 and Figure 1. Radioactivity was present in CO_2, aspartate, glutamate, glutamine, and GABA in decreasing order. The greatest amount of radioactivity was present in CO_2 and aspartate in all age groups, yielding more than 84% of the total radioactivity. Taking the total recovered radioactivity of 5-week-old rats as 100%, those of 10, 55, and 80-week-old rats were 111.4%, 115.6% and 122.2%, respectively. The aspartate uptake in the growing period (5 and 10 weeks of age) remained low, whereas that in the aging period (55 and 80 weeks of age) increased significantly ($P < 0.05$). The same trend was observed in the syntheses of glutamate, glutamine, and GABA, although the CO_2 production showed

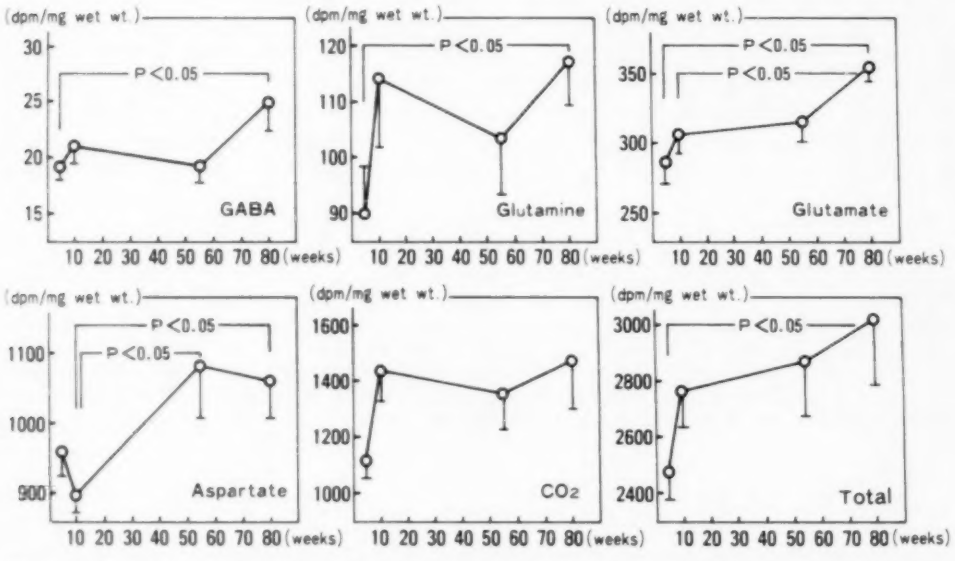

FIG. 1. Radioactivity of aspartate and its metabolites obtained from the rat cerebral cortical slices of varying ages (mean + S.E.M.).

TABLE 1. RADIOACTIVITY OF ASPARTATE AND ITS METABOLITES FROM THE RAT CEREBRAL CORTICAL SLICES (DPM/MG WET WT.)

Age	*GABA*	*Gln*	*Glu*	*Asp*	CO_2	*Total*
5 weeks (n = 13)	18.9[a]	89.3[a]	286.7[a]	961.1	1123.4*	2482.3[a]
	±1.2	±7.7	±16.9	±34.6	±74.1	±109.4
10 weeks (n = 12)	21.0	114.3	306.4[b]	899.9[b,c]	1424.3	2766.5
	±1.5	±11.9	±14.7	±27.8	±106.2	±130.5
55 weeks (n = 16)	19.0	103.6	316.4	1083.1[c]	1347.1	2869.0
	±1.6	±10.2	±12.7	±70.8	±129.7	±212.2
80 weeks (n = 12)	24.6[a]	117.5[a]	354.8[a,b]	1062.7[b]	1473.5*	3032.8[a]
	±2.3	±7.8	±10.2	±52.7	±169.1	±230.3

Cerebral cortical slices obtained from varying ages were incubated with (U-^{14}C)L-aspartate at 37°C for 45 min. The results are mean + S.E.M. Abbreviations: GABA-γ-aminobutyrate, Gln-glutamine, Glu-glutamate, Asp-aspartate.

a-a, b-b, c-c: statistically significant at $P<0.05$, respectively. *-*: $P<0.10$.

a gross tendency to increase during aging. Consequently, the overall aspartate metabolism in the rat parietal cortex was enhanced in the aging period when compared to the growing period.

DISCUSSION

Changes in neurotransmitter amino acids with aging are ambiguous in the literature. De Koning-Verest (1980) has found a 13% decrease in aspartate and glutamate concentrations, but not in glutamine in rat brains. According to Timiras *et al.* (1973), the glutamate and alanine of rats between 2 and 30 months of age were not statistically reduced in the cortex, whereas aspartate declined in the cortex (−35%). Tyce and Wong (1980) have observed reduction in glutamate (−9%) and glutamine (−15%) concentrations in aged rats. Rajeswari and Radha (1984) have reported that the levels of glutamate, glutamine, and aspartate decreased with age, whereas those of GABA and alanine increased in the whole brains of rats. In brains of aged mice, an increase in glutamine (Kirzinger and Fonda, 1978) and a decrease in GABA (Fonda *et al.*, 1973) have been reported. On the other hand, Gibson *et al.* (1981) have found no concentration changes of aspartate, glutamate, glutamine, GABA, alanine, and serine in the whole brains of aged rats.

The measurement of the dynamics of synthesis of these neurotransmitters is necessary in order to clarify the above discrepancies. In that connection, some in vivo and in vitro studies are available. Tyce and Wong (1980) have discovered a significant reduction of carbon from glucose to glutamate, aspartate, and alanine in the cerebrum of 22-month-old rats. Gibson *et al.* (1981) have reported that aspartate was the only amino acid in which carbon incorporation from glucose declined significantly by 10 months of age in rats. We have also recently demonstrated that overall (U-^{14}C)D-glucose oxidation in the parietal cortex declined but the metabolic pathway to form amino acids was not uniformly suppressed in 78-week-old rats (Matsumoto *et al.*, to be published).

In the present study we found that the aspartate uptake as well as the syntheses of glutamate, glutamine, and GABA from aspartate increased in the parietal cortex of 80-week-old rats. Using the same in vitro method employed here, we previously reported a highly significant reduction of glutamate uptake with markedly decreased conversion of gluta-

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mate to aspartate in the parietal cortex of 100-week-old rats (Matsumoto *et al.*, 1982), whereas in the present study there was an increase of aspartate uptake with fairly constant conversion of aspartate to glutamate (11.0–11.7%) in all age groups. These findings support the idea that the dynamic metabolism of aspartate is definitely different from that of glutamate, although both amino acids are excitatory neurotransmitters (Johnson, 1978) and appear to be transported by the same uptake mechanism in brain slices (Balcar and Johnston, 1972). The age-related increases in the activities of glutamate dehydrogenase (Victoria *et al.*, 1981) and aspartate aminotransferase (Oeriu *et al.*, 1962) appear to be reasonable for supporting the concept of enhanced synthesis of glutamate. We observed an increase in glutamine formation. This would strongly suggest that ammonia fixation mechanism in the brain is functioning well with enough compensatory capacity up to 80 weeks of age, because the activity of glutamine synthetase in the brains of rats ages 1, 2, 12, and 24 months has been reported to be almost the same (Gordienko, 1975). Recently, Rajeswari and Radha (1984) have demonstrated an age-dependent increase of glutamate decarboxylase (GABA forming enzyme) and a decrease of GABA-transaminase (GABA degrading enzyme) in rat brains. Their observation is a possible explanation for the increased GABA incorporation. However, the increase of GABA could be related to the vulnerability to anoxia encountered during slice preparation of the parietal cortex, because experimental evidence indicates that brain ischemia causes an increase of GABA associated with a simultaneous increase of glutamate decarboxylase and decrease of GABA-transaninase activity (Mršulja *et al.*, 1978). The CO_2 production tended to be increased in the parietal cortex at 80 weeks of age. The primary source of CO_2 in our study is believed to be from the Krebs cycle, because aspartate is converted into glutamate therein (Simon *et al.*, 1967) and the GABA shunt metabolism is said to be around 8% (Balázs *et al.*, 1970; Baxter, 1976). Therefore, the increasing trend of CO_2 production with aging supports the hypothesis that aspartate metabolism in the brain is enhanced at least up to 80 weeks of age.

Judging from aspartate uptake and conversion in the parietal cortex, aspartate metabolism during the aging period was more active than in the growing period. This may suggest that enzyme activities involved in aspartate metabolism have not yet reached a fully functioning state in growing brains.

SUMMARY

Aspartate metabolism was studied in parietal cortical slices from male Wistar rats ages 5, 10, 55, and 80 weeks. The slices were incubated with (U-^{14}C) L-aspartate in O_2-saturated Gey's balanced salt solution. Subsequently, the radioactivity levels of liberated CO_2, aspartate, and its metabolites (glutamate, glutamine, and GABA) were obtained from these slices. The greatest amount of radioactivity was recovered from CO_2 and aspartate, which yielded more than 84% of the total radioactivity. Aspartate uptake in the growing period (5 and 10 weeks of age) remained low, whereas that during the aging period (55 and 80 weeks of age) increased significantly. Essentially the same results were observed in the syntheses of glutamate, glutamine, and GABA, although CO_2 production showed a tendency to increase. Consequently, overall aspartate metabolism was enhanced during the aging period compared to that of the growing period. The above findings support the hypothesis that aspartate metabolism in the brain accelerates with advancing age, at least up to 80 weeks of age.

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INFLUENCE OF FASTING AND REFEEDING ON THE ELONGATION STEP OF PROTEIN SYNTHESIS IN RAT LIVER OF YOUNG (3M) AND AGING (18M) ANIMALS

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Abstract—The in vitro binding capacity for AA-tRNA of ribosomes, catalysed by EF_1 enzyme, was studied in livers of rats, which had been starved for 5 days and re-fed for 3, 9, and 24 hours. The experiments were carried out with 3 and 18-month-old rats. The results were compared to those obtained with normally fed rats of the same ages. For both age groups, there was a similar change in the binding capacity, including a return to normal values after 24 hours of refeeding. When ribosomes of old control animals were treated with different enzymes, we obtained lower binding activities for all the experimental groups than in homologous cell-free systems (ribosomes and EF_1 isolated from the same group). The ribosomes of young animals, tested in the presence of different enzymes, display an increased binding capacity, when the enzymes used were those of old (control and re-fed) animals. The recovery of the catalytic activity of EF_1 on the binding process was observed after 9 hours in old animals, whereas it was not seen for young re-fed animals at that time of refeeding. EF_1 seems to play an important role in the adaptation of older animals to fasting and refeeding.

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INTRODUCTION

AGING IS a complex of various phenomena, one of these being the reduced capacity to adapt to changes in the environment (Adelman, 1971, 1979). Protein metabolism is one of the major processes in which adaptation capacity is altered. Conflicting results have been obtained in different studies on protein synthesis in aging animals, depending on the species, strain, age, organ, and biological state of the animal and the experimental methods used (Makrides, 1983; Rothstein, 1982).

Adaptation of protein synthesis to various experimental circumstances, including the aging process, can occur by changes in the elongation step (Bolla and Brot, 1975; Moldave *et al.*, 1979; Vargas and Castañeda, 1981; Webster and Webster, 1982; Gabius *et al.*, 1983). A previous study (Roels-De Schrijver, 1982) showed that in rat liver the capacity for older animals to respond to the stress of fasting diminishes and that alterations of ribosomes and mRNA are probably responsible for this phenomenon.

This confirms the results of Breuer and Fiorini (1965), Britton and Sherman (1974), Kurtz (1978), Ekström *et al.* (1980), Cook and Buetow (1981) and Vandenhaute *et al.* (1983) on liver, muscle, and brains of rodents, and of Wallach and Gershon (1974) on nematodes.

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The present study compares the recovery of protein synthesis in young (3 months) and aging (18 months) rats during 24 hours of refeeding after an initial 5 days of fasting. By combining two of the essential elements of protein synthesis (ribosomes and EF_1*) from different experimental groups in an in vitro system, we have tried to unravel some aspects of the adaptation of protein synthesis to a specific stress situation. We conclude that older animals can adapt the elongation step to refeeding after fasting as efficiently as young ones, albeit by different pathways.

MATERIALS AND METHODS

Animals

Male rats of the Lewis strain, with a mean life span in laboratory breeding of about 24 months, were used throughout the experiments. The animals were kept in cages (four rats/cage) with a bedding of wood shavings. The cages were cleaned 3 times a week. At regular intervals an autopsy was performed on apparently healthy individuals to check any respiratory infection that could spread among the colony. The animals were maintained at normal laboratory temperature and outdoor light cycles. They were fed a normal diet ad libitum (16% proteins; 34% carbohydrates; 2.5% fat; vitamin A, D, B, PP, E, and choline) and had free access to water. During the experiments the animals were kept in separate cages and a constant temperature of 21°C. At least four rats were used for each group; all were killed at the same time of day. Before processing, the livers of all animals within the same group were pooled. We considered two age groups: a group of 18-month-old animals and one of 3-month-old animals. Each group was subdivided into five sets of at least four rats, corresponding to five different experimental conditions. A control set from each age group was fed a normally balanced stock diet, which was also used with refeeding. The other rats were subjected to fasting for a period of 5 days. A set of both old and young rats was killed after this period. The remaining sets were re-fed for 3, 9, or 24 hours, respectively, before killing.

Preparation of subcellular fractions

Ribosomes were prepared from quickly removed and chilled livers according to the method described by Clemens and Pain (1974), with some modifications. The livers were homogenized in a Potter-Elvehjem (B. Braun, Melsungen, Frg.) in buffer H (20 m*M* Tris-HCl pH 7.5; 4 m*M* magnesium chloride; 250 m*M* sucrose; 6 m*M* β-mercapto ethanol). After 10 minutes of centrifugation at 10,000 *g*, the supernatant was saved, while avoiding the upper lipid layer present at the top of the tube in control animals or animals re-fed for 9 hours or more. The supernatant was treated with Triton X-100 and magnesium chloride at final concentrations of 1% and 0.1 *M* respectively, at a temperature of 4°C for the duration of 1 hour. Fifteen ml suspension was layered on 15 ml buffer G (20 m*M* Tris-HCl pH 7.5; 20 m*M* magnesium chloride; 400 m*M* sucrose; 6 m*M* β-mercapto ethanol) in R_{30} Spinco centrifuge tubes (Ânalis, Namur, Belgium), and spun for 15 minutes at 27,000 *g*. The supernatant was very carefully aspired, and the loose pellet was suspended in Medium S (20 m*M* Tris-HCl pH 7.5; 100 m*M* potassium chloride; 40 m*M* sodium chloride; 5 m*M* magnesium chloride and 6 m*M* β-mercapto ethanol).

At this stage, according to Clemens and Pain (1974), the preparation consists mostly of polyribosomes. These "crude" polysomes were further treated with ammonium chloride at a final concentration of 0.5 *M*, according to the method of Moldave and Skogerson (1967), in order to remove enzymes involved in protein synthesis.

This treatment, however, resulted in a complete reduction of endogenous mRNA activity in a cell-free system. Every new batch of "stripped" ribosomes was checked for endogenous mRNA activity. Only batches without endogenous messenger activity, and completely dependent on the presence of polyuridylic acid as synthetic messenger in the cell-free system, were retained for further studies. After treatment with 0.5 *M* ammonium chloride, the ribosomes were suspended in Medium S and stored in liquid air.

EF_1 (Elongation factor 1) was isolated from the pH 5 supernatants of the postribosomal fraction, according to the method of Felicetti and Lipmann (1968). This protein fraction, precipitated between 25% and 75% saturated ammonium sulphate, and containing EF_1, was dialysed against a buffer *M* (50 m*M* Tris-HCl pH 7.5; 250 m*M* sucrose; 0.1 m*M* EDTA; 2 m*M* glutathione and 15% glycerol). The enzymes were stored at −20°C.

Binding assay

The activities of EF_1 and ribosomes were quantitated by counting the radioactivity of the ^{14}C-phenylalanyl-tRNA-polyuridylic acid-ribosome complex, retained on nitrocellulose filters (Weissbach *et al.*, 1973). The formation of the complex is catalysed by EF_1.

*EF_1: eukaryotic elongation factor 1

Assay mixtures (500 μl) contained 100 μg of stripped ribosomes, 100 μg of *E. coli* tRNA charged with ^{14}C-phenylalanine (specific activity 504 mCi/mMol); 100 μg polyuridylic acid; 200 μg EF_1 in buffer (Tris-HCl 50 m*M* pH 7.4; dithiotreitol 5 m*M*; ammonium chloride 160 m*M*; magnesium chloride 20 m*M*; guanosine triphosphate 0.5 m*M*). This reaction is totally dependent on the presence of polyuridylic acid (100 μg) and GTP (0.5 μ*M*). It is linear for at least 40 minutes at 37°C. Reactions were stopped after 30 minutes by adding 2 ml washing buffer (Tris-HCl 0.5 *M* pH 7.5; potassium chloride 0.014 *M*; magnesium chloride 0.008 *M*). Radioactivity was measured in a Berthold scintillation counter (± 85% Effic.) after solubilization of the nitrocellulose membrane in Hydroluma (Lumac, Basel, Switzerland). The activity was expressed in counts per minute per mg ribosomal RNA.

In order to compare the activities of different groups in separate experiments, the binding results were expressed in percentages of values obtained simultaneously in a cell-free system with ribosomes and EF_1 of young control animals. All these figures have been tested statistically. The differences were tested by Student's *t* test. The rejection of the null-hypothesis was fixed at the limit of $P = 0.02$.

Materials

^{14}C-phenylalanine (500 mCi/mole) was obtained from the Radiochemical Center (Amersham, England); tRNA from *E. coli*, polyuridylic acid (poly U), and guanosine triphosphate (GTP) were purchased from Serva (Heidelberg, F. R. Germany). Hydroluma was purchased from Lumac System (Basel, Switzerland).

RESULTS

The binding activity of the EF_1 was measured in a homologous cell-free system containing the ribosomes and EF_1 of the same set under saturating conditions. Table 1 indicates only minor differences between the binding capacities of two control groups. The difference between young and old animals is most pronounced after 5 days of fasting, but the recovery pattern during the first 24 hours after refeeding runs practically parallel in the two age groups, with an increase in the binding capacity after 3 hours of refeeding, followed by a sharp decline after 9 hours and an almost complete recovery after 24 hours.

The *t* test shows that the difference between the binding activities of homologous cell-

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TABLE 1. (^{14}C) PHENYLALANYL-tRNA BINDING IN A HOMOLOGOUS SYSTEM (RIBOSOMES AND EF_1 OF SAME EXPERIMENTAL GROUP)

Age	*Experimental condition*	*Relative binding activity**
3 months		
	Control**	100
	Fasted	83 ± 4.5 (3)
	Fasted (5d) Re-fed (3hrs)	140 ± 14.5 (3)
	Fasted (5d) Re-fed (9hrs)	60 ± 13.7 (5)
	Fasted (5d) Re-fed (24hrs)	105 ± 18.6 (5)
18 months		
	Control	91 ± 16.8 (5)
	Fasted	43 ± 5.5 (3)
	Fasted (5d) Re-fed (3hrs)	122 ± 6.1 (3)
	Fasted (5d) Re-fed (9hrs)	62 ± 22.4 (3)
	Fasted (5d) Re-fed (24hrs)	91 ± 20.5 (3)

* Relative binding activity is expressed as percentage of the activity obtained with ribosomes and EF_1 from young control animals, simultaneously processed. All results are given as mean ± s.e.m., with the number of experiments given in parenthesis.

** The results obtained for the control group (3 months) in c.p.m./1mg ribosome RNA is 1.091 ± 101 (7).

Binding activities in a homologous cell-free system of young and old fasting animals are significantly different at $P < 0.01$, on Student's *t* test.

free systems of matching experimental age groups is not significant. In fasting animals, however, the *t* test shows a significant difference between the age groups of 3 and of 18 months.

In a heterologous cell-free system the results are more divergent when ribosomes of control animals of different ages were combined with the EF_1 of different experimental sets. When ribosomes from young control rat livers were combined with EF_1 of young fasting rats (Table 2A) we note a significant increase in the elongation process. The restoration of the binding capacity after refeeding young animals is about the same as in the homologous system, a decline of the activity after 9 hours of refeeding and a return to normal values after 24 hours. With the EF_1 of old control and fasting rats used in combination with young ribosomes, we notice an important improvement in the binding capacity of these young ribosomes. The recovery pattern is somewhat different from the one observed in the previous systems. Here an earlier decline in the binding capacity occurs as soon as 3 hours after refeeding. This is restored after 9 hours. After 24 hours of refeeding, the binding capacity of the rat liver ribosomes of young animals recovers to the values obtained in the presence of young ribosomes and control "old" EF_1. When rat liver ribosomes of

TABLE 2. (^{14}C) PHENYLALANYL-tRNA BINDING IN A HETEROLOGOUS SYSTEM (RIBOSOMES AND EF_1 OF DIFFERENT GROUPS)

A. *Ribosomes of young (3month) animals*	*EF_1*	*Relative binding activity*
3 months	Control*	100
	Fasted (5 days)	168 ± 23.5 (5)
	Fasted (5d) Re-fed (3hrs)	162 ± 17 (6)
	Fasted (5d) Re-fed (9hrs)	68 ± 8 (5)
	Fasted (5d) Re-fed (24hrs)	112 ± 6 (5)
18 months	Control	181 ± 19
	Fasted (5 days)	174 ± 12 (6)
	Fasted (5d) Re-fed (3hrs)	98 ± 9 (7)
	Fasted (5d) Re-fed (9hrs)	119 ± 18 (5)
	Fasted (5d) Re-fed (24hrs)	171 ± 26 (5)
B. *Ribosomes of old (18month) animals***	*EF_1*	*Relative binding activity*
3 months	Control	46 ± 10 (5)‴
	Fasted (5 days)	42 ± 17 (6)‴
	Fasted (5d) Re-fed (3hrs)	53 ± 7 (6)‴
	Fasted (5d) Re-fed (9hrs)	34 ± 13 (6)‴
	Fasted (5d) Re-fed (24hrs)	45 ± 17 (7)‴
18 months	Control	74 ± 24 (6)‴
	Fasted (5 days)	44 ± 14 (6)‴
	Fasted (5d) Re-fed (3hrs)	32 ± 9 (5)‴
	Fasted (5d) Re-fed (9hrs)	48 ± 13 (5)‴
	Fasted (5d) Re-fed (24hrs)	57 ± 27 (5)‴

*The results obtained for the control group (3months) (Ribosomes and EF_1) is 1065 c.p.m./1 mg ribosome RNA ± 65 (4)

**Significance determined by paired *t* test A. versus B.

‴ $P < 0.02$; ‴ $P < 0.01$; ‴ $P < 0.001$;

old animals are present in the cell-free system (Table 2B), the stimulation of binding with EF_1 of young control animals is lower than with EF_1 of old control animals and the effect of EF_1 from young and old fasting animals is similar.

Although all of the values obtained in a heterologous cell-free system containing ribosomes of old rats are below the 100% value of the control group, the general evolution of binding activities during refeeding is the same as that of the heterologous cell-free system that included the ribosomes of young rats. The decline and return to normal values with the EF_1 of old animals is faster than that observed with the EF_1 of young animals.

The *t* test shows significant differences in the binding capacities of heterologous cell-free systems of corresponding experimental sets, performed with ribosomes of either 3- or 18-month-old rats.

DISCUSSION

It is generally accepted that protein synthesis can be modulated at the translational level. Changes in mRNA, ribosomes, and soluble factors can be responsible for this either independently or simultaneously. In reticulocyte systems the initiation step can play an important role in the rate of protein synthesis, as is shown by Lodish (1976) and Hunt (1980). The complexity and the number of factors involved in protein initiation make the interpretation of the results of these in vitro studies very difficult. Protein synthesis can also be modulated at the elongation step, as mentioned by Pain and Clemens (1973), and confirmed by several other studies, such as in cultured cells (Vero cells by Hassel and Engelhardt in 1976, and Hela cells by Nielsen and McConkey in 1980), in fish liver during acclimation to cold by Nielsen *et al.* (1977), in the immune spleen by Willis and Starr (1971), in the kidney after $AgCl_2$ (Nicholls and Ng, 1976) and $CdCl_2$ (Kuliszewski and Nicholls, 1980), in the liver of the cockerel after β-estradiol (Gehrke *et al.*, 1981), as well as in the liver of the rat after DDT (Young and Nicholls, 1978), after methylmercury (Sauvé and Nicholls, 1981) and after fasting and refeeding (Roels-De Schrijver, 1974).

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The changes in elongation rate observed could be attributed to factors such as changes in aminoacyl-tRNA, messenger RNA, EF_1, and the ribosomes themselves. We limited ourselves to the study of the ribosomes and EF_1 in the first step of the cycle: the binding of phenylalanyl-tRNA to the acceptor site of the ribosome.

The treated ribosomes used in our binding experiments were devoid of endogenous mRNA. This was replaced by polyuridylic acid, so that changes in messenger activities cannot account for variations in the binding capacity. Observations on protein synthesis in aging animals obtained by several investigations are difficult to evaluate (Rothstein, 1982; Makrides, 1983). Differences in life span between species, and even between strains, individuals, sexes, and the conditions of life make comparison of results an arduous task.

Aging does not influence different organs of the same animal in a similar way (Blazejowski and Webster, 1983). Moreover, the response to aging is not always a simple decrease in metabolic functions, because biphasic response to aging, starting with a sharp decline of protein synthesis followed by an increase, is observed as well in liver cells of mice (Claes-Reckinger *et al.*, 1982) and rats (Van Bezooyen *et al.*, 1977). We are aware that our choice of two age groups doesn't provide any information about the evolution of protein synthesis after fasting and refeeding between these two periods of the animals' life. Therefore, it is necessary to extend our research to other age groups. Variations in hepatic protein synthesis result in part from subtle changes in the hormonal influences on

the liver. Age-dependent alterations of the hepatic functions could reflect modifications of extra hepatic hormonal mechanisms (Adelman, 1979). As is mentioned by Rothstein (1982), there can be a substantial degree of loss of function in older organisms without great harm. It is rather the adaptation to stress that seems to be impaired with aging (Makrides, 1983). By submitting old and young animals to the extreme situation of fasting and refeeding, we tried to detect possible failures in the adaptation of protein synthesis. Previous experiments (Roels-De Schrijver, 1972) have shown that fasting followed by a short period (3 hours) of refeeding stimulates the elongation process in livers of 3-month-old rats. The stimulation may be due to the soluble fractions, as well as to the polysomes. Fasting not followed by refeeding has also a stimulating effect in the binding capacity of liver polysomes of young animals as compared to 12-month-old fasting adults. These polysomes preincubated and devoid of endogenous mRNA (run-off ribosomes) displayed a less marked, but analogous, effect of age on the binding activity (Roels-De Schrijver, 1982). Variations in the number of active ribosomes or in the accessibility of binding sites on the ribosomes could account for the greater binding of ribosomes in young fasting animals.

Following the binding capacity in the elongation step in vitro during the first 24 hours of refeeding after fasting, we observed a similar recovery pattern in young and old animals, although old fasting animals have a lower binding rate than young fasting ones (Table 1). We conclude that old and young animals have the same ability to restore the binding step in protein synthesis after 5 days of fasting. In in vitro heterologous binding experiments with rat liver ribosomes from either young or old control animals together with EF_1 isolated from different experimental sets, we tried to find which factor is responsible for the recovery of the binding step of protein synthesis during refeeding. The decreased binding efficiency of senescent ribosomes observed with EF_1 under different experimental conditions is noteworthy. (Table 2B vs. 2A) It confirms the results of Britton and Sherman (1975), Ekström *et al.* (1980), Cook and Buetow (1981) and Roels-De Schrijver (1982) about age-related alteration of the ribosomes or their proteins, resulting in a decreased interaction with aminoacyl-tRNA. Kurtz (1978) attributed the decline of the ribosomal activity to a diminution of the number of active ribosomes in the cell. On the other hand, in a recent study, Horbach *et al.* (1984) found the protein synthesizing capacity of free and membrane-bound polyribosomes and of cell sap unchanged in young and senescent female rats. They objected to certain separation procedure of ribosomes. They argued that the isolated ribosomes mostly are not representative for the entire ribosome population.

Shmookler-Reis (1981), studying cell-free protein synthesis of aging liver ribosomes of individual mice, reported an impairment of the ribosomal activity only in particular individuals. In most of the animals he observed a regular increase with age of the synthesizing capacity of the ribosomes together with an improved response to poly U. It is noteworthy that the animals of this study were fed a nutritionally supplemented diet, whereas our animals were not. This difference in nutritional status can affect the protein synthesizing capacity of the ribosomes, as emphasized by Shmookler-Reis himself. It is striking that EF_1 isolated from old control animals as well as from old rats re-fed 24 hours has a higher binding capacity than the enzyme of young animals (Table 2). This increase probably accounts for the analogous recovery pattern in young and old rats, despite altered ribosomes in aging animals. It may seem peculiar that the binding capacity of EF_1 is higher in aging control and recovered (24 hrs) rat livers, because an age-related decline in protein synthesis is, at least partly, attributed to a decrease in EF_1 activity (Moldave *et al.*, 1979; Gabius

et al., 1983). It is likely that the quality of EF_1 of young and old animals is different. It has been reported that eukaryotic EF_1 studies may provide an explanation for this observation. According to Legocki *et al.* (1974), multiple forms of EF_1, isolated from various tissues, have different characteristics. The light, monomer form (EF_{1L}) should react more efficiently than the heavy aggregated form (EF_{1H}), which represents a storage form of the enzyme. Bolla and Brot (1975) have noticed a conversion of high molecular weight enzymes in young nematodes to a low molecular weight form in aging nematodes.

In *A. salina*, a shift of EF_{1H} to EF_{1L} is observed during the development from cysts to nauplii (Slobin and Möller, 1975). In aging rats subjected to fasting and refeeding, an analogous shift from heavy to light EF_1 could take place and affect the rate of translation. The possibility that EF_{1H} of older organisms is more easily broken down during isolation cannot be ruled out. In that case, it could be an indication for changes in composition of EF_{1H}.

The recovery pattern during refeeding in an in vitro binding reaction in a homologous system is about the same for ribosomes and enzymes obtained from livers of either young or old rats. However, the mechanism for this adaptation is different. By comparing the activity of EF_1 of different origin in the presence of ribosomes of both young and old animals, we have observed that a decrease of activity in senescent ribosomes can be compensated by an increase of EF_1 activity in old rat livers. Other factors in protein synthesis that have not been investigated in the present study, such as initiation and dissociation factors, as well as mRNA, can change with age and influence the ultimate protein synthesizing capacity. Although a compensatory mechanism for the binding step in the elongation of proteins in vitro seems to exist, the question remains as to the efficiency of the compensation for the synthesis of the proper proteins in vivo, and how long it can be maintained in an aging organism undergoing stress.

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AGE-RELATED DIFFERENCES IN RESPONSE TO ACUTE COLD CHALLENGE (−10°C) IN MALE F344 RATS

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Abstract—Male F344 rats, 3, 12, and 24 months of age, were acclimated to either 5°C (CA) or 23°C (non-CA). At the end of acclimation, all CA rats exhibited higher basal oxygen uptake than the non-CA when measured at 28°C. When challenged by a 3-hour exposure to −10°C, significant difference in tolerance to cold was observed not only between the CA and non-CA rats but among the different age groups. The 3-month-olds tolerated cold better than 12-month-olds, and the 12-month-olds did better than the 24-month-olds. The ranking order for cold tolerance in −10°C was: 3-month CA > 12-month CA > 3-month non-CA = 12-month non-CA = 24-month CA > 24-month non-CA. Total 3-hour O_2 uptake decreased progressively with advancing age. Heat productions were doubled in −10°C in all age groups, and all CA rats had higher heat production than non-CAs. The increase of heat production in −10°C was insufficient to maintain body temperature in all animals. All animals had loss of body heat content, the loss becoming greater with advancing age. Non-CA rats had greater loss of heat content or faster decline of colonic temperature than the CAs. Significant strain-related difference in cold tolerance was noted between the F344 and Sprague-Dawley (S-D) rats, because 24-month-old non-CA male F344 rats still exhibited a better tolerance to −10°C than 3-month-old CA S-D rats.

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INTRODUCTION

COLD ACCLIMATION to a 5°C environment enhances rats' tolerance to more intense cold, as this has been demonstrated by better maintenance of heat production and colonic temperature in cold-acclimated (CA) rats than in non-cold-acclimated (non-CA) rats when challenged by a transient exposure to −10°C (Kiang-Ulrich and Horvath, 1979, 1980). This effect appeared to be generally similar in different strains of male rats studied, such as Sprague-Dawley (S-D) and Fischer 344 (F344), although F344 exhibited significantly enhanced tolerance to cold compared to S-D, whether cold acclimated or not (Kiang-Ulrich and Horvath, 1984). However, age-related differences in cold tolerance, either in cold-acclimated or non-cold-acclimated rats, have not been determined. We recently described age-related differences in responses to chronic 5°C cold exposure in male F344 rats (Kiang-Ulrich and Horvath, 1985). We now present data demonstrating age-related differences in male F344 rats to a 3-hour −10°C acute cold challenge with and without prior cold acclimation to 5°C.

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METHODS AND MATERIALS

Male F344 rats* (3, 12, and 24 months of age) were maintained in either 23°C (non-CA) or 5°C (CA) environments (six in each age group) for 7 to 9 weeks and with a 12-hour light/dark cycle. Purina Lab Chow and water were provided ad libitum. During the last week of acclimation all rats were subjected to a cold tolerance test, that is 3 hours at −10°C. Oxygen uptakes and colonic temperatures were monitored throughout the test period. Body weights were taken before and after the test.

Oxygen uptake was measured in a constant-volume closed system (Volume Meter, model 160, Med Science, Needham, MA) with the rat in a sealed plexiglass chamber and expired carbon dioxide absorbed by soda-lime. Two constant-temperature baths were utilized to maintain chamber temperatures of 28°C or −10°C. Colonic and chamber temperatures were measured continuously with thermistor probes and recorded on a dual-channel recorder.

Oxygen uptakes and colonic temperatures were initially determined at 28°C to obtain baseline values. The animal was then transferred to the −10°C chamber. Measurement of oxygen uptakes and colonic temperatures were continued for 3 hours.

Total oxygen uptakes in 180 minutes were calculated. Heat productions and heat contents were calculated as follows:

$$\text{Heat production} = [\text{body weight (kg)} \times \text{metabolic rate (ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}) \times 0.005]$$
$$\text{Heat content} = [\text{body weight (kg)} \times \text{colonic temperature (°C)} \times 0.83]$$

All data were evaluated by an analysis of variance and covariance and followed by ad hoc comparisons (Tukey) where significant main effects and interactions were found. Decreases of colonic temperature were analyzed by a series of linear regressions across time to determine the best-fitting curve. The significance of the differences between regression coefficients (slopes) was determined by using Student's t test. The 0.05 level of significance was selected for rejection of the null hypothesis.

RESULTS

There was no significant difference in the body weights between the 12- and 24-month-olds measured before the test, whether in CA or non-CA rats. The 12- and 24-month-old animals weighed significantly more than the 3-month-olds. In general, CA rats weighed less than non-CA rats, but this difference was only statistically significant for the 3-month-olds. All rats had significant weight loss during the cold tolerance test, but the difference between groups remained the same as observed before the test (Table 1).

Basal O_2 uptake of animals acclimated to 23°C and measured at thermoneutral temperature (28°C) decreased insignificantly as age advanced: 20.9 ml·kg^{-1}·min^{-1} for 3-month-olds, 18.4 for 12-month-olds, and 17.4 for 24-month-olds. Cold acclimation resulted in significant increases in basal metabolism measured at 28°C in all age groups: that is, 24.6 (18% increase) for the 3-month-olds, 24.3 (32% increase) for the 12-month-olds, and 21.8 (25% increase) for the 24-month-olds. The age-related difference remained the same in CA animals, that is, basal O_2 uptake decreased (though insignificantly) as age advanced (Table 1).

Exposure to the −10°C environment prompted an immediate increase in O_2 uptake in all animals. Cold-acclimated rats responded with a significantly higher initial increase and had higher peak values than non-CA rats of the same age groups. Among the age groups, 3-month-olds had significantly higher initial increases than the 12- and 24-month-olds, CA or non-CA. There was no significant difference in the initial responses between the 12-and 24-month-old animals. At the end of 3 hours the difference in O_2 uptake between

*In conducting this research, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee for Laboratory Animal Facilities and Care, of the Institute of Animal Laboratory Resources, National Academy of Sciences–National Research Council.

TABLE 1. BODY WEIGHTS, O_2 UPTAKES, AND COLONIC TEMPERATURES

	Body weight		*O_2 uptake*				*Colonic temperature*		
	(g) ± S.E.		*ml·kg⁻¹·min⁻¹ ± S.E.*			*ml·kg⁻¹ in 180 min ± S.E.*	*°C ± S.E.*		*°C·min⁻¹*
Grouping of male rats	*Before test*	*After test*	*Basal at 28°C*	*Immediate increase in −10°C*	*At end of 180 min in −10°C*	*Total 180 min in −10°C*	*Basal at 28°C*	*At end of 180 min in −10°C*	*Rate of decrease in −10°C (β)*
3-mo, non-CA	322 ± 4.5*†‡	305 ± 4.0*†‡	20.9 ± 0.9*	47.5 ± 0.7*†‡	40.2 ± 1.6*‡	8349 ± 100*†‡	37.1 ± 0.1	30.4 ± 0.6*‡	0.0395*‡
3-mo, CA	266 ± 7.4*†‡	249 ± 6.9*†‡	24.6 ± 0.9*	63.5 ± 1.6*†‡	51.2 ± 3.3*†‡	10489 ± 146*†‡	37.6 ± 0.2	34.5 ± 0.2*‡	0.0142*†‡
12-mo, non-CA	376 ± 7.3†	359 ± 7.2†	18.4 ± 1.2*	37.1 ± 0.9*†	38.0 ± 0.6§	7313 ± 59*†§	37.4 ± 0.2	30.7 ± 1.8*§	0.0390*§
12-mo, CA	365 ± 7.8†	346 ± 6.6†	24.3 ± 0.8*	41.0 ± 1.2*†	43.5 ± 0.8†	8070 ± 128*†§	37.4 ± 0.1	34.3 ± 0.4*§	0.0196*†§
24-mo, non-CA	385 ± 13‡	368 ± 13.2‡	17.4 ± 1.0*	35.7 ± 1.1*‡	25.8 ± 1.6*‡§	6129 ± 165*‡§	37.4 ± 0.2	26.2 ± 1.2*‡§	0.0664*‡§
24-mo, CA	362 ± 10.5‡	343 ± 11‡	21.8 ± 0.8*	41.3 ± 1.4*‡	37.5 ± 4.6*‡	7329 ± 296*‡§	37.3 ± 0.2	30.6 ± 1.0*‡§	0.0385*‡§

Symbols: * significantly ($P < 0.05$) different between CA and non-CA
† " " " " 3-mo and 12-mo
‡ " " " " 3-mo and 24-mo
§ " " " " 12-mo and 24-mo

CA and non-CA remained significant in the 3- and 24-month-olds. The age-related differences were significant between the 3- and 12-month-old CA, the 12- and 24-month-old non-CA, and between the 3- and 24-month-old CA and non-CA animals (Table 1, Figure 1).

Total O_2 uptakes for the 3-hour cold exposure are presented in Table 1. The 3-month-olds, CA or non-CA, had the largest total uptakes, with the 12- and 24-month-old values being significantly less. The 24-month-olds had the smallest total O_2 uptake of the three age groups studied. All CA rats had higher total O_2 uptakes than non-CA in each age group (Table 1).

Basal colonic temperatures at 28°C were not significantly different related to either age or acclimation. During the −10°C challenge, colonic temperatures fell at different rates in different age and acclimation groups (Figure 2, Table 1). Non-CA rats had a significantly more rapid rate of decline than CA rats in each age group. Young 3-month-old rats had significantly slower rate of decline than the 12- and 24-month-olds. Our ranking orders of the rates of decline are: 3-month CA < 12-month CA < 3-month non-CA = 12-month non-CA = 24-month CA < 24-month non-CA. Mean colonic temperatures at the end of 3 hours followed the same pattern (Table 1). The rankings for cold tolerance are: 3-month CA > 12-month non-CA > 3-month non-CA = 12-month non-CA = 24-month CA > 24-month non-CA. Heat production was increased but the body heat content was reduced in −10°C from those in 28°C in all animals. Cold-acclimated animals had higher heat productions and smaller reduction of body heat content in −10°C than the non-CAs. The

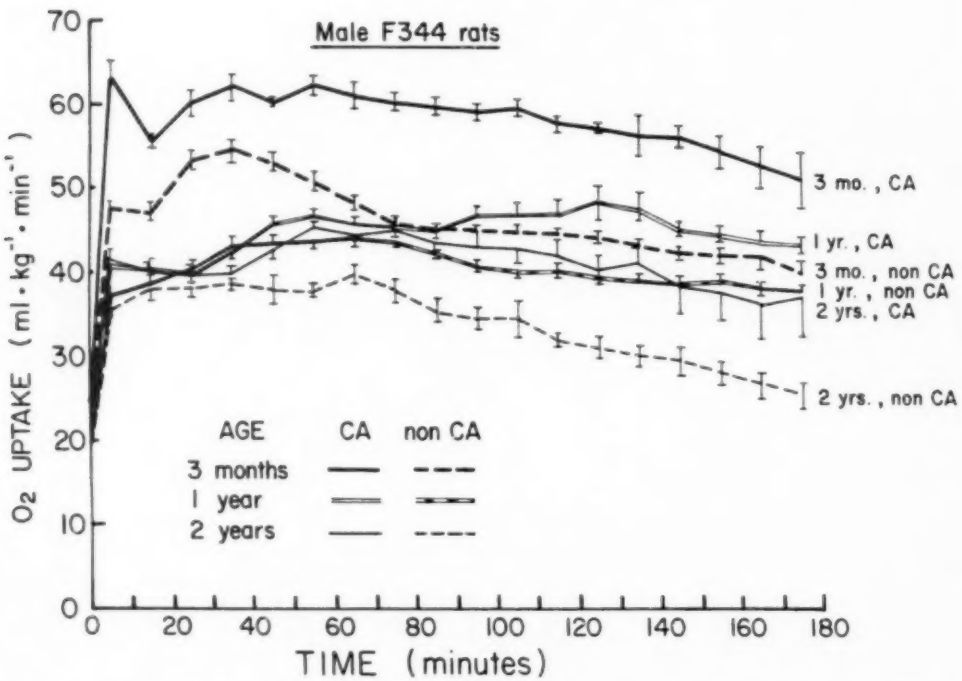

FIG. 1. Oxygen uptakes during 3-hour −10°C exposure. Each data point represents average O_2 uptake (ml·kg^{-1}·min^{-1}) in each 10-min period, mean ± S.E. Six animals in each group.

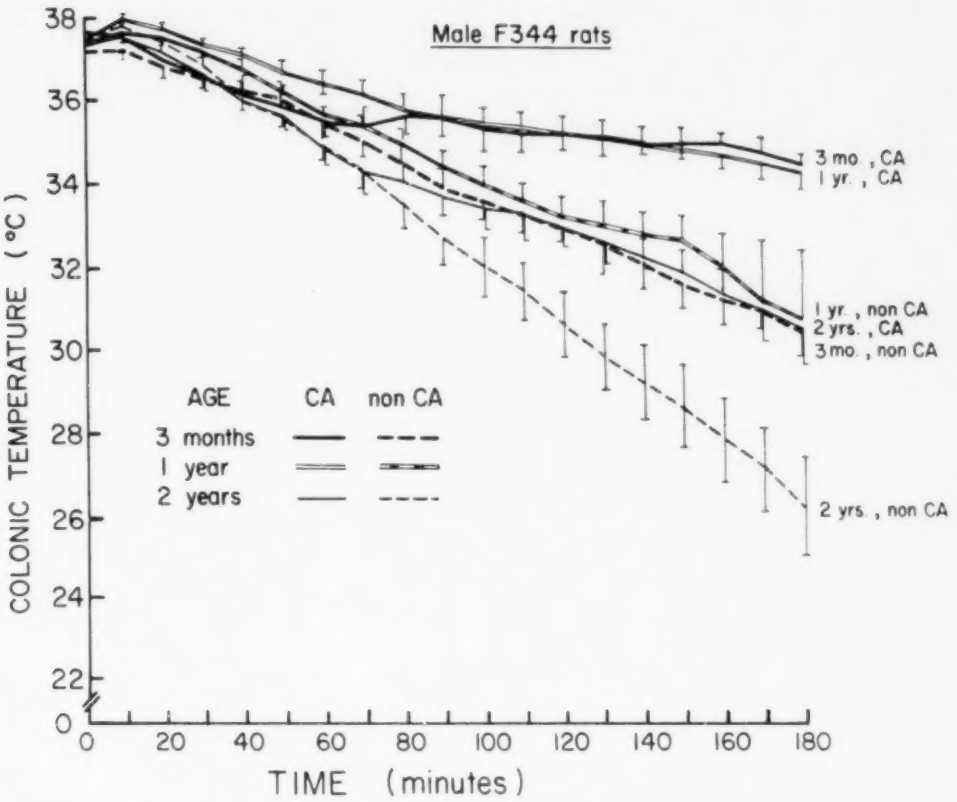

FIG. 2. Colonic temperature (°C) during the 3-hour −10°C exposure. Each data point represents mean ± S.E. at end of each 10-min period. Six animals in each group.

12-month-olds had higher (though insignificant) heat production and greater reduction of body heat content than the 3-month-olds (significant in CA animals only), but the 24-month-olds had significantly smaller heat production and significantly greater reduction of body heat content than the 12-month-olds in −10°C whether CA or non-CA (Figure 3, Table 2).

DISCUSSION

Tolerance to −10°C was clearly enhanced by prior cold acclimation (5°C) in all age groups of male F344 rats. This was evidenced by higher immediate increase, peak, and total oxygen uptake with slower rate of decrease and better maintenance of colonic temperature in the cold-acclimated animals than in the non-cold-acclimated. As rats grew older their response to the cold (−10°C) challenge deteriorated. Thus 3-month-olds tolerated −10°C better than 12-month-olds and 12-month-olds did better than the 24-month-olds. The interaction of age and acclimation was evidenced by the ranking order of cold tolerance presented in the results.

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TABLE 2. HEAT PRODUCTION AND HEAT STORAGE

Grouping of male F344 rats	*Heat production kcal ± S.E. (during 180 min)* 28°C	−10°C	*Body heat content kcal ± S.E. at end of 180 min* 28°C	−10°C	*Δ body heat content kcal* −10°C
3-mo, non-CA	6.0 ± 0.2	13.0 ± 0.2‡	9.9 ± 0.1*†‡	7.7 ± 0.2†	−2.3 ± 0.1*‡
3-mo, CA	6.0 ± 0.2†‡	13.5 ± 0.4	8.3 ± 0.2*†‡	7.2 ± 0.2†‡	−1.1 ± 0.1*†‡
12-mo, non-CA	6.2 ± 0.4*	13.4 ± 0.2*§	11.7 ± 0.2†	9.2 ± 0.6†	−2.5 ± 0.4§
12-mo, CA	7.5 ± 0.3*†§	14.3 ± 0.2*§	11.3 ± 0.2†	9.8 ± 0.2†§	−1.5 ± 0.1†§
24-mo, non-CA	6.0 ± 0.3*	11.5 ± 0.3*‡§	12.0 ± 0.4‡	8.0 ± 0.5	−3.9 ± 0.4*‡§
24-mo, CA	7.0 ± 0.2*‡§	12.8 ± 0.3*§	11.2 ± 0.3‡	8.5 ± 0.3‡§	−2.5 ± 0.3*‡§

Symbols: * significantly ($P < 0.05$) different between CA and non-CA
† " " " " 3-mo and 12-mo
‡ " " " " 3-mo and 24-mo
§ " " " " 12-mo and 24-mo

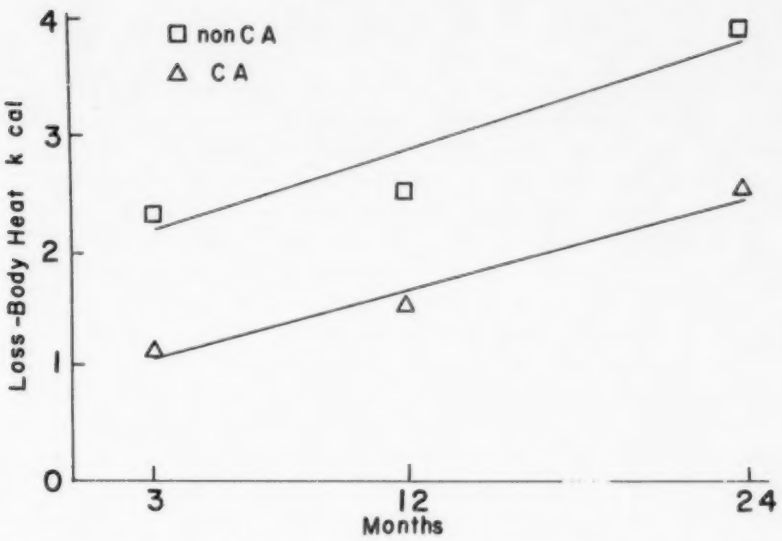

FIG. 3. Relationship of body heat loss in −10°C to age (3, 12, and 24 months) and acclimation. Six animals in each group.

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The 24-month-old non-CA rats appeared to have the lowest O_2 uptake (25.8 ml·kg^{-1}·min^{-1}) (Table 1), the lowest percentage increase in O_2 uptake (48%) (Table 3), and the lowest colonic temperature (26.2°C) (Table 1) at the end of 3 hours as compared to all the other age and acclimation groups. Although we did not objectively measure shivering activity, there is little question that they must have been shivering initially. However, as their colonic temperature fell dramatically, they reached the level at which shivering no longer is present. Blatteis and Horvath (1958) have shown that shivering in rodents disappears as a regulatory factor when colonic temperature reached 32–31°C. Consequently it must be assumed that the inability of these older animals to maintain a high O_2 uptake resulted in further heat loss and a further reduction in colonic temperature.

Heat production was essentially doubled in −10°C in all groups, but this marked increase was insufficient to maintain body heat content. All animals had increased heat storage losses from their bodies, being greater as the animal aged regardless of whether

TABLE 3. PERCENTAGE INCREASES IN O_2 UPTAKES

	Non-CA		*CA*	
Age group (mo.)	*Immediate*	*End of 3 h*	*Immediate*	*End of 3 h*
3	127	92	158	108
12	100	106	69	79
24	105	48	89	72

they were acclimated to either 23°C or 5°C. However, the 23°C acclimated rats all had greater storage losses than the 5°C acclimated in all groups.

The 3-month-old CA had the highest total oxygen uptake of all groups in −10°C. However, the calculated heat production of 3-month-old CA was less (though insignificant) than that of the 12-month-old CA. The significantly slower rate of decrease in colonic temperature in 3-month-old CA might have been the result of a significantly smaller reduction of body heat storage—the smallest of all groups. Although 12-month-old CA had the highest heat production in −10°C, the body heat content loss was significantly greater than that of 3-month-old CA; thus cold tolerance was reduced. Without benefit of prior cold acclimation, the 12-month-old non-CA had higher (insignificant) heat production but also greater (insignificant) body heat content loss than the 3-month-old non-CA, resulting in similar cold tolerance in 3- and 12-month-old non-CA animals. The 24-month-old CA were able to improve cold tolerance to a similar degree as 3-month-old and 12-month-old non-CA with a similar heat production and a similar heat content loss as the 3- and 12-month-old non-CA rats. The 24-month-old non-CA rats had the lowest cold tolerance of all groups, clearly due to a lower heat production (the lowest of all groups) and a greater heat content loss (the greatest of all groups) than the younger animals. The body heat content loss appeared to play an important role in all groups (Figure 3, Table 2).

It is worth noting that the 12-month-old male F344 rats appeared to be more efficient in energy economy than the 3-month-olds, CA or non-CA. The 12-month-olds had a significantly smaller initial rise of oxygen uptake immediately after −10°C exposure than the 3-month-olds, significantly smaller total O_2 uptake in 3 hours than the 3-month-olds, but had a greater (though insignificant) heat production in −10°C than the 3-month-olds (Tables 1 and 2). We have noted a similar efficiency in energy economy in this age group during cold acclimation, as smaller increases in food intake were required to maintain body weight than for the 3- and 24-month-olds (Kiang-Ulrich and Horvath, 1985). This efficiency in energy economy could be due to enhanced responsiveness in brown adipose tissue (BAT). BAT has been identified as the principal site of non-shivering thermogenesis (NST) in rodents (Foster and Frydman, 1978) and exerts an important role in buffering energy balance (Rothwell and Stock, 1979). However, most BAT information was obtained from Sprague-Dawley, Long Evans, Holtzman, and Wistar rats. No studies on F344 have been reported. We speculate that there well may be some significant difference in responsiveness in BAT among different age and acclimation groups.

Because differential body heat content loss appeared to have an important role in cold tolerance, sympathetic nervous function must be involved. Although the role of norepinephrine (N-E) in the trophic response of BAT heat production to cold has been questioned (Mory *et al.*, 1978), direct sympathetic influence on vasoconstriction and hence body heat content loss must be considered. An increase of sensitivity to catecholamines of a number of effectors alongside with certain weakening of sympathetic influences upon the organs and tissues in aging rats have been observed. However, there were variable changes in the sensitivity vessels of various parts to catecholamine administration and to sympathetic influences in aging (Frolkis *et al.*, 1970). Studies on sympathetic influence on vasoconstriction in aged rats may explain the increased degree of body heat content loss with aging in −10°C.

Interestingly, the 24-month-old non-CA male F344 rats, which showed the least cold tolerance of all groups, still exhibited a better tolerance than the 3-month-old Sprague-Dawley rats. The 3-month-old S-D had a colonic temperature of 24.7°C at the end of

170 min. The rate of decrease in colonic temperature was 0.0697°C·min^{-1}. These rats did not finish the 180 min test time (Kiang-Ulrich and Horvath, 1984). It is apparent that significant differences in tolerance to extreme cold occur in different strains of rats. These differences raise important questions as to the interpretation of data obtained on different strains of research animals.

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THE EFFECTS OF AGING AND CHRONIC DIETARY RESTRICTION ON WHOLE BODY GROWTH AND PROTEIN TURNOVER IN THE RAT

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Abstract—Changes in whole body growth, nucleic acids, and protein turnover have been studied in conjunction with ageing and chronic dietary restriction. Normal developmental changes between weaning and senescence included progressive decreases in the fractional rates of growth, protein synthesis, and protein breakdown; the decline in the synthetic rate correlating with decreases in the ribosomal capacity. Dietary intervention was imposed at weaning and involved pair feeding to 50% of the ad libitum food intake. Although this regime slowed whole body growth by retarding the developmental decline in protein turnover, growth was extended into the second and third years of life. The dietary-induced increase in longevity resulting from a retardation of the ageing process(es) appears therefore to be associated with an enhanced turnover of proteins during the major portion of the life span of dietary restricted rats.

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INTRODUCTION

AGEING IS an inevitable characteristic of life. The numerous theories concerning ageing (Shock, 1977; Comfort, 1979) can be broadly divided into genetic and nongenetic groups. The latter group attributes senescence to a general "wear and tear" of existing cellular proteins and structures. The former theories assume that the ageing process is programmed by DNA, which is susceptible to mutations, less efficient repair, and increased incidence of errors in the subsequent steps of transcription and translation. A useful animal model for studying the process of ageing is the dietary-restricted rodent. Although it is well established that chronic dietary restriction appreciably increases the life span of the rat (Barrows and Kokkonen, 1977; Ross, 1978; Merry and Holehan, 1979, 1981), the mechanisms by which the increased longevity occurs remain obscure. Chronic underfeeding is, however, known to effectively reduce the frequency of spontaneous tumours and postpones the onset of many age-related diseases, for example, pneumonia and nephrosis (Ross, 1978); thus supporting the epiphenomenalist theories. However, dietary restriction may also influence cellular ageing by altering the rate of DNA replication and the subsequent

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efficiency of transmission of the genetic information into cellular proteins (Barrows and Kokkonen, 1978; 1980), that is, is consistent with the fundamentalist theory.

In the present investigation the developmental changes in whole body growth in the dietary restricted rat have been studied from weaning through to senescence. These events have been compared with the growth of littermate controls of the same sex, which were allowed to feed ad libitum. Emphasis has been placed on explaining the observed changes in body growth by defining the alterations in protein turnover, both as a function of age and dietary restriction.

MATERIALS AND METHODS

Male Sprague-Dawley (CFY strain) rats were used throughout this study. All animals were weaned at 21 days and randomly allocated to control or experimental groups, where they were caged individually and allowed free access to water. Dietary-restricted rats were pair fed to 50% of ad libitum fed controls (Rat Breeder diet, E.H. Bradshaw & Sons Ltd., Driffield, England; Merry and Holehan, 1979, 1981). Whole body protein synthesis was measured in vivo 10 min after an intravenous injection of a large dose of phenylalanine to flood the precursor pools (Garlick *et al.*, 1980; Goldspink and Kelly, 1984). This injection was administered via a lateral tail vein and contained 150 μmol of the amino acid, including 65 μCi of *L*-[4-^{3}H] phenylalanine (sp. radioactivity 24 Ci/mmol; from Amersham International P.L.C., Amersham, Bucks, U.K.) in 1 ml of 0.9% NaCl per 100 g body weight. Animals were killed by decapitation, exsanguinated (15 s) and their abdominal and thoracic cavities opened immediately prior to the whole animal being frozen in liquid N_2.

Whole bodies were pulverized (between chilled metal plates) in a coldroom and then homogenized in 0.3 M $HClO_4$ (1:4 w/v) in a Waring blender. Several (three to five) 10 ml samples of this preparation were then further homogenized in a ground-glass homogenizer before analysis. The good agreement (i.e., within 8% of the mean) between these three to five samples attested to the homogenous sampling of the whole-body preparations. The specific radioactivities of the phenylalanine both in the "flooded" tissue pool(s) and covalently bound in protein were measured in the homogenates of the whole bodies, by the method described by Garlick *et al.*, 1980. This involved the prior hydrolysis of the washed protein pellets in 6 M HCl at 110°C for 24 h and the conversion of phenylalanine into β-phenethylamine. All measurements of radioactivity were made in a LKB scintillation counter (efficiency of 25% for tritium) in a Triton X-100/xylene-based scintillant with the use of an external standard. The fractional rate of synthesis (i.e., k_s, the percentage of the protein mass synthesized per day) was calculated from

$$k_s = \frac{S_B}{S_A t} \times 100$$

where S_A and S_B are the specific radioactivities of phenylalanine in the free tissue pool and protein, respectively, and t is the time in days.

Proteins were measured on the same tissue or whole-body preparations by the method of Lowry *et al.* (1951), using bovine serum albumin (Sigma, Kingston upon Thames, Surrey, U.K.) as standard. RNA and DNA were also extracted and measured as previously described (Goldberg and Goldspink, 1975). Growth rates (k_g) were measured as the net accumulation of protein in the whole bodies between two time intervals close to, or spanning, the point at which synthesis rates were measured. The exact time intervals varied, being as short as possible in situations of rapid growth (e.g., 2 days), and becoming longer (i.e., 7 days) as growth slowed; precise details being presented in the relevant tables. Growth rates (k_g) were then expressed as the percentage change in the protein content per day. Because growth arises from an imbalance in the rates of synthesis (k_s) and degradation, protein breakdown (k_b) was calculated, by subtraction, from the two measured parameters, that is, $k_b = k_s - k_g$.

RESULTS

Various parameters associated with growth were measured in control and dietary-restricted rats at different stages of their lives, that is, at weaning, both pre- and post-pubertal phases and in senescence (Table 1). From weaning (22 days) to senility (721 days) the body weight and protein mass of *ad libitum* fed controls increased approximately

TABLE 1. THE EFFECTS OF AGE AND CHRONIC DIETARY RESTRICTION ON WHOLE-BODY PROTEIN AND NUCLEIC ACID CONTENTS

Age (days)		*Body wt. (g)*	*Total protein (g)*	*Protein/ wet wt. (%)*	*Total RNAP (mg)*	*RNAP/ wet wt. (μg/g)*	*Total DNAP (mg)*	*DNAP/ wet wt. (μg/g)*	*RNAP / DNAP*
22									
	Zero time control	59 ± 2	7.2 ± 0.2	12.1 ± 0.3	17.8 ± 0.4	301 ± 6	9.4 ± 0.4	158 ± 3	1.91 ± 0.04
52									
	Control	264 ± 6	42.1 ± 1.0 (+488^{x})	16.0 ± 0.6	66.3 ± 1.5 (+271^{x})	252 ± 8	33.5 ± 1.0 (+256^{x})	126 ± 2	1.99 ± 0.06
	Restricted	87 ± 1	12.5 ± 0.4 (+73^{x}) [−70^{x}]	14.3 ± 0.4	18.4 ± 0.6 (+3 NS) [−72^{x}]	211 ± 6	10.6 ± 0.3 (+12^{xx}) [−68^{x}]	121 ± 3	1.75 ± 0.07
365									
	Control	547 ± 43	95.0 ± 5.0 (+1219^{x})	17.5 ± 0.7	68.1 ± 2.8 (+282^{x})	126 ± 8	39.2 ± 1.9 (+317^{x})	73 ± 7	1.75 ± 0.06
	Restricted	239 ± 15	46.5 ± 2.9 (+545^{x}) [−51^{x}]	19.5 ± 0.5	41.1 ± 1.1 (+131^{x}) [−39^{x}]	172 ± 7	19.7 ± 0.2 (+109^{x}) [−49^{x}]	83 ± 5	2.08 ± 0.04
721									
	Control	604 ± 13	72.6 ± 3.1 (+908^{x})	12.3 ± 0.7	67.4 ± 2.2 (+278^{x})	114 ± 6	29.9 ± 1.2 (+218^{x})	50 ± 3	2.31 ± 0.06
	Restricted	347 ± 12	53.6 ± 2.9 (+644^{x}) [−26^{x}]	15.5 ± 1.4	53.3 ± 2.4 (+199^{x}) [−21^{x}]	155 ± 8	19.2 ± 1.8 (+104^{x}) [−35^{x}]	56 ± 4	2.64 ± 0.05
1042									
	Control								
	Restricted	358 ± 16	52.1 ± 1.3 (+623^{x})	14.6 ± 0.3	53.5 ± 1.0 (+200^{x})	150 ± 5	23.8 ± 0.8 (+153^{x})	67 ± 1	2.26 ± 0.04

Frozen control or dietary-restricted whole bodies were chopped into small pieces by the use of a guillotine and subsequently pulverized between chilled metal plates. The powdered bodies were then allowed to thaw in ice-cold 0.3 M − $HClO_4$ (1 : 4, w/v) before being homogenized in a Waring blender at 3°C. Three to five samples (10 ml) of this homogenate were withdrawn and further homogenized in a motor-driven ground-glass homogenizer. Proteins were measured in these fine homogenates by the method of Lowry *et al.* (1951), and RNA and DNA were extracted and measured as described by Goldberg and Goldspink (1975). The values obtained from the three to five separate samples were then averaged to give a single value for each animal. Each value at each age is the mean ± S.E.M. for three or four control, or dietary-restricted rats treated as described above. Values in ( ) parentheses give the percentage changes with respect to zero controls, and values in [ ] brackets the percentage differences between age-matched control and dietary-restricted rats. Student's *t* test ($^{xx}P < 0.05$, $^{x}P < 0.01$, was used to determine the level of statistical significance.

TABLE 2. THE EFFECTS OF AGE AND CHRONIC DIETARY INTERVENTION ON WHOLE-BODY PROTEIN TURNOVER.

Age (days)		*Fractional rate of synthesis (%/day)*	*Total protein synthesized*			*RNAP/ protein (mg/g)*	*Growth rate (%/day)*	*Calculated rate of breakdown (%/day)*
			(g/day)	*(g/day per kg body wt)*	*(g/day per g of RNAP)*			
22								
	Zero time control	32.2 ± 0.9	2.31 ± 0.1	38.9 ± 1.6	129 ± 5	2.5 ± 0.96	6.7	25.5
52								
	Control	26.5 ± 1.3 (−17^{xx})	11.12 ± 0.3 (+382^{x})	42.1 ± 2.0 (+8)	168 ± 5 (+29^{x})	1.6 ± 0.05 (−36^{x})	4.7	21.8
	Restricted	25.9 ± 1.5 (−19^{x}) [−2]	3.24 ± 0.5 (+40^{xx}) [−70^{x}]	37.2 ± 5.9 (−5) [−11]	180 ± 10 (+39^{xx}) [+7]	1.5 ± 0.03 (−40^{x}) [−6]	1.8	24.1
365								
	Control	9.3 ± 1.8 (−71^{x})	8.94 ± 1.9 (+287^{x})	16.3 ± 3.7 (−58^{x})	127 ± 24 (−2)	0.7 ± 0.02 (−72^{x})	1.2	8.1
	Restricted	13.5 ± 1.0 (−58^{x}) [+45^{xx}]	6.29 ± 0.6 (+172^{x}) [−29]	26.4 ± 2.6 (−32^{x}) [+61^{xx}]	153 ± 13 (+18) [+20]	0.9 ± 0.03 (−64^{x}) [+23^{xx}]	0.6	12.9

(Table 2 continues)

(Table 2 continued)

721								
	Control	10.8 ± 1.3	7.86 ± 1.5	13.1 ± 0.7	117 ± 16	0.9 ± 0.03	0.1	10.9
		(−66^{x})	(+240^{x})	(−66^{x})	(−9)	(−64^{x})		
	Restricted	11.5 ± 3.9	5.98 ± 1.8	17.2 ± 5.0	115 ± 41	1.0 ± 0.06	0.2	11.2
		(−64^{x})	(+158^{x})	(−55^{x})	(−10)	(−60^{x})		
		[+5]	[−24]	[+31]	[−2]	[+6]		
1042								
	Control							
	Restricted	12.6 ± 1.3	6.54 ± 0.57	18.3 ± 2.3	122 ± 11	1.0 ± 0.01	0.22	12.4
		(−61^{x})	(+183^{x})	(−53^{x})	(−5)	(−60^{x})		

Protein synthesis was measured in the whole bodies of three to four control or dietary-restricted rats 10 min after injecting 150 μmol of phenylalanine (containing 65 μCi of the ^{3}H-labelled amino acid) per 100 g of body wt, via a lateral tail vein. The exsanguinated bodies were rapidly frozen and subsequently homogenized (see Table 1). The fractional rate of protein synthesis was then calculated after determining the specific radioactivities of both the free and protein-bound phenethylamine in the whole bodies (see METHODS section). The total amount of protein synthesised per day was then calculated for each animal as the product of its fractional rate and protein content (Table 1). This value was then divided by either the body weight or the RNA content obtained from Table 1. Growth rates were determined as the daily percentage increase in the protein mass of the whole animal; changes in the protein contents being measured over 2, 5, or 7-day intervals in the 52, 365, and 720-day-old rats, respectively. The fractional rate of protein breakdown was also calculated, by subtracting the measured rates of growth from those of synthesis. Values in ( ) parentheses indicate the percentage changes relative to zero control values, whereas figures in [ ] brackets represent the percentage changes between age-matched control and dietary-restricted rats. Significance levels were determined using Student's t test ($^{xx}P < 0.05$, $^{x}P < 0.01$).

10-fold, whereas in the dietary-restricted animals (maintained on 50% of the food intake of the controls) these increases were significantly lower at approximately 6-fold and 8-fold, respectively. Although the protein mass generally increased with age, the growth rate (i.e. the daily percentage increase in the protein mass) progressively decreased (Table 2) reaching steady state conditions at 721 days. Although the same was basically true after dietary intervention, growth rates were, age for age, lower than in the ad lib fed control animals, even though a slow rate of growth persisted between 2 and 3 years (Table 2). The generally lower rate of growth in the dietary-restricted rats appeared to arise primarily from a decrease in cell number (i.e., total DNA; Table 1) rather than any significant change in cellular hypertrophy (i.e., protein/DNA). That is, the developmental increase in protein/DNA (from 0.77 ± 0.03 to 2.4 ± 0.02 at 22 and 721 d, respectively) was expressed equally in both groups of rats. However, during the same time the accumulation of DNA, and hence cellular proliferation, was markedly suppressed (35–70%) in the dietary restricted animals (Table 1) in agreement with Merry and Holehan (1985).

For the most part the nucleic acid ratios were unchanged during normal development and in conjunction with dietary restriction (Table 1). Although RNA and DNA still increased after dietary intervention, the accumulation of both nucleic acids was equally suppressed, thereby giving rise to smaller age-related gains compared with those found in the ad libitum fed animals. These situations were however altered slightly in senescence. In the old control rats between 1 and 2 years there was a small (23%) but significant ($P < 0.005$) decrease in DNA, without any change in the RNA. This small loss of DNA effectively caused the nucleic acid ratio to increase. Although a similar increase in this ratio was seen in the old dietary-restricted rats (at 2 and 3 years) this arose by different means, that is, via a proportionately greater increase in RNA than DNA (Table 1), but without any net loss of either nucleic acid during this period of slow growth (Table 2).

To explain the changes in whole body growth in response to ageing and dietary manipulation, protein synthesis was measured in the whole animal after an injection of a large dose of ^3H-phenylalanine (Garlick *et al.*, 1980; Goldspink and Kelly, 1984). The fractional rate of synthesis (i.e., the percentage of the protein mass renewed per day) was found to decrease from 32% at weaning (i.e., 22 days) to 9–11% between 1 and 2 years (Table 2). A similar developmental pattern emerged in the dietary-restricted animals. To take into account the increasing size of the animals the total amount (g) of protein synthesised per day (i.e., protein mass × fractional synthetic rate) was also calculated. This initially increased with age in the controls; between 22 and 52 days, the increasing protein mass of the growing animals more than compensating for the decreasing fractional rate of synthesis. However, at the ages studied thereafter (i.e., 1 and 2 years) progressively less protein was synthesised, whether expressed as g/day or g/kg body wt/day; the latter simply allowing for differences in animal size. Dietary manipulation did slightly reduce the total amount of protein (g/day) synthesised at each age, with the most profound effect being evident early after its imposition (i.e., 52 days). However, when allowances were made for the smaller size of the calorie-restricted animals (i.e., per kg weight) synthetic rates were approximately 30–60% higher in these rats, compared with controls at 721 and 1042 days (Table 2).

Because the rate of protein synthesis in any cell depends on its number of ribosomes and the activity of those ribosomes in the translation process, it is often useful to examine such parameters in parallel. An index of ribosomal capacity in the whole animal can be obtained from RNA/protein values, whereas ribosomal activity can be gauged from synthesis rates

expressed per unit RNA (Henshaw *et al.*, 1971). From such information it is apparent that the decline in the fractional rates of synthesis during normal postnatal life parallels decreases in the ribosomal capacity (i.e., RNA/protein; Table 2). In similar manner the lower total rates of synthesis (g/day; Table 2) at the various stages of dietary restriction correlated with the presence of less cellular RNA (Table 1). In contrast, little or no discernable differences existed in ribosomal activities (i.e., synthesis/RNA; Table 2), during development in either control or dietary-restricted rats: the major exception to this generalization being the acute phase of dietary intervention (i.e., 52 days).

The final parameter to be considered in explaining the altered rates of growth during normal development and dietary manipulation concerns the rate of protein breakdown (Table 2). This, like synthesis, has been shown to be under physiological control. For technical reasons, it is difficult to obtain meaningful direct rates of protein breakdown in vivo, for example, the nonexponential decay of prelabeled proteins and the efficient reutilization of amino acids precludes accurate measurements of degradation. Hence the rates presented here are indirect values calculated from the measured rates of synthesis and growth. Although this represents the most accurate method currently available to us (Garlick, Fern and McNurlan, 1979), these indirect rates of protein breakdown are presented as indicators of the probable changes in whole body protein degradation. For example, the large overall decrease in these values in control animals between weaning and senescence is likely to indicate a genuine developmental decline in protein breakdown, as well as protein synthesis (Table 2). Protein degradation was also marginally, but consistently, increased, along with the fractional rate of synthesis, at each age of dietary intervention. These smaller differences in breakdown, as appear to arise from the reduced food intake, should however be viewed with more caution.

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In this type of study it is essential to distinguish between the immediate (i.e., daily) and long term (i.e., weekly) effects of modifying food supply, particularly when measuring nutritionally sensitive parameters of growth such as protein synthesis. That is, protein turnover in several body tissues is known to be dependent upon the timing of the last meal relative to the measurement of synthesis or breakdown (Waterlow, Garlick and Millward, 1978). In contrast to the Fischer 344 rats used by Liepa *et al.* (1980), by 1 year of age the CFY rats in this study had adapted to eating their smaller daily food rations over a full 24 hours. Hence in this investigation potential problems arising from piecemeal feeding would only be encountered at the earliest time of 52 d. Here, the animals still ate their food ration within a few hours of its presentation, thereafter having to await the next daily supply. This could, and did, have an appreciable bearing on some of the measured values associated with growth at 52 days.

The effects of this piecemeal feeding at the 52 d stage were therefore investigated on three groups of dietary-restricted rats. All animals were littermates and had been dietary-restricted since weaning (see previous). The only differences between the three groups of rats was the timing of their last meal, that is, 3, 12, or 23 h, before making the measurements associated with growth. To ensure that the timing of the presentation of food was the only variable, protein synthesis was consistently measured between 9:00 and 11:00 a.m. (as at all other ages in this study) so as to avoid possible complications arising from diurnal variations. Although the protein content of the rats fed 23 h prior to death was slightly lower than those of animals fed only 3 h before (Table 3), the body weights and nucleic acid contents of these animals were not significantly different (data not shown).

TABLE 3. CHANGES IN WHOLE BODY PROTEIN TURNOVER IN 52-DAY-OLD DIETARY-RESTRICTED RATS IN RESPONSE TO THE TIMING OF THEIR LAST MEAL

Time after last meal (h)	*Total protein (g)*	*Fractional rate of synthesis (%/d)*	*Total protein synthesized*	
			(g/day)	*(g/day per g of RNAP)*
3	12.5 ± 0.4	25.9 ± 1.5	3.24 ± 0.46	176 ± 10
12	12.1 ± 0.3	22.2 ± 1.1	2.68 ± 0.31	149 ± 15
	(−3)	(−14^{xx})	(−17)	(−15^{xx})
23	10.7 ± 0.2	17.0 ± 1.5	1.82 ± 0.14	97 ± 8
	(−14^{x})	(−34^{x})	(−44^{xx})	(−45^{x})

All analytical procedures and measurements were the same as described in Tables 1 and 2. Protein synthesis was measured 3, 12, or 23 h after food was last presented to the 52-d-old (i.e., 30 d calorie-restricted) rats. Each value is the mean ± S.E.M. of three or four dietary-restricted rats, with values in parentheses representing the percentage changes relative to the measurements made 3 h after feeding. ($^{xx}P < 0.05$, $^{x}P < 0.01$).

However, both the fractional and total rates of protein synthesis, and the ribosomal efficiency (i.e., synthesis per RNA), changed; the decreases becoming greater the longer the time interval between the last meal and the measurement of protein synthesis (Table 3).

These findings clearly indicate the importance of diet on whole body protein turnover and the need to isolate acute dietary effects from the chronic influences on growth. Consistent with this, the data presented in Tables 1 and 2 for the 52-day-restricted animals are those measured 3 h after the last meal, hence eliminating problems associated with piecemeal feeding. At all other ages studied here the dietary-restricted animals had completely adapted to this regime such that they, like the controls, had access to food at all times over the full 24 h period.

DISCUSSION

For many years considerable interest has been directed towards understanding the effects of starvation or malnutrition on protein turnover in man and animals (Read *et al.*, 1971, 1972; Young *et al.*, 1973; Felig and Wahren, 1974; Goldspink, 1978). However, the majority of these studies have been of a short-term nature attempting to define the effects of calorie or protein restriction over only a few days or weeks. This study is fundamentally different in that the effects of chronic food (calorie) restriction have been examined at various ages from weaning to senescence. Such long-term dietary intervention has previously been shown to be an effective way of prolonging life span in the rat (Sacher, 1977; Comfort, 1979; Merry and Holehan, 1979, 1981). However, a significant but smaller increase (10–20%) in rodent life span can be achieved by initiating underfeeding at one year of age, (Weindruch and Walford, 1982) where a controlled loss of body mass accompanies the gradual adaptation to the restricted diet. When restricted feeding is implemented throughout postweaning, life extension in life span is correlated with a slower, more protracted growth of the whole animal (Table 2). The most profound effects of this type of chronic dietary manipulation occurred early after its imposition, that is, in the 52 d-old-animals where rates of growth and protein turnover are normally high (Tables 1 and

2). The slowing of growth in the dietary-restricted rats may possibly arise primarily from a decrease in cell number (total DNA; Table 1) (Merry and Holehan, 1985). This can only be stated with some caution however, because not all of the body's cell types are mononucleate. In particular, skeletal muscle is multinucleate and the whole musculature normally undergoes very rapid growth during postnatal life (Lewis *et al.*, 1984). Because the DNA of the musculature constitutes some 30–35% of whole body DNA, any preferential slowing of growth and nuclear proliferation in muscle could have an important bearing on the accuracy of this index of cell number. Because of the difficulties in obtaining biopsy specimens, most of the available information on protein turnover in man comes from whole body studies. However, in addition to gaining access to individual tissues, studies on the rat are particularly useful in that these animals represent a relatively homogeneous population that can be subjected to the same environmental conditions and diet. Furthermore, the rat possesses a conveniently short life span and studies on the effect of chronic nutritional intervention can be carried through to senescence with relative ease. Even so, to our knowledge, the effects of altered nutrition on developmental changes in protein turnover throughout the life span of an animal have not been undertaken.

The fractional rate of synthesis in the whole body of the ad libitum fed controls progressively decreased with age (Table 2). This is in agreement with the developmental changes in the synthetic rates in other strains of the rat (Waterlow *et al.*, 1978; Goldspink and Kelly, 1984). A similar developmental pattern to that of the controls was apparent in the dietary-restricted animals, but in keeping with the more prolonged period of growth the age-related decline in protein turnover was more gradual in these animals, that is, the synthetic and degradative rates in the restricted rats were slightly higher at both 365 and 721 days. Our findings basically agree with Picou and Taylor-Roberts (1969), who indicated that the rates of protein turnover when measured in malnourished children were higher than when subsequently determined during their recovery when provided adequate nutrition. Whole body protein degradation has also been reported to decrease after an increased protein intake (Golden *et al.*, 1977).

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Because the ingestion of protein, and possibly other dietary components, probably exerts short-term influences on the rate of protein synthesis in certain body tissues (Waterlow *et al.*, 1978), the effect of feeding the same diet 3 or 23 h before injection with a radioactive tracer was investigated. Predictably, the actual time of eating within a 24 h cycle did not significantly alter the whole body weights, or DNA and RNA contents. However, the fractional rate of protein synthesis did decrease as a function of time since the last meal (Table 3). The reductions in protein synthesis measured in the rats 12 and 24 h after feeding probably reflects the decreased rates of synthesis known to occur in certain tissues, for example, liver, gut, and muscle; when starvation is imposed over short periods (Goldspink, 1978; Rannels *et al.*, 1978; Waterlow *et al.*, 1978). Clague and colleagues (1983) also found that whole body protein synthesis in humans decreased in response to acute fasting. These acute reductions in whole body protein synthesis can be linked to decreases in ribosomal efficiency (i.e., synthesis/RNA; Table 3). However, in the absence of such daily, nutritionally-related fluctuations in protein turnover a chronic increase in protein degradation, rather than any change in synthesis, (Table 2) probably accounts for the slower rate of growth at 52 d (Table 2). Therefore, at all other ages, where the dietary-restricted animals had nutritionally adapted, both the fractional rates of synthesis and breakdown were consistently higher than in the ad libitum fed control animals.

In the dietary-restricted rat model, where chronic underfeeding is maintained throughout postweaning life, a retardation of the normal progressive decrease in protein turnover is observed and somehow, as yet undefined, this is linked to an increased longevity. It is not known if a similar effect on protein turnover occurs in rodents whose life span is extended as a result of underfeeding during adult life only. Mitogen-induced protein synthesis and IL-2 production are known to be increased by dietary restriction in 19-month male Fischer F344 rats (Cheung *et al.*, 1983). Similarly, at 19 months of age protein synthetic activity in cell-free homogenates from testis, spleen lymphocytes, and kidney cells has also been shown to be significantly increased by dietary restriction, (i.e., 60% restriction) of Fischer F344 rats as compared with animals fed ad libitum (Sparks *et al.*, 1983). Hence the data presented here on whole body rates of protein synthesis and degradation (i.e., measured in vivo) in dietary-restricted rats support these results from studies on cells in vitro and cell-free systems (Cheung *et al.*, 1983; Sparks *et al.*, 1983). Richardson and Cheung (1982) have proposed that the decline in protein synthesis and degradation (turnover) that occurs with increasing age would provide a molecular basis for the physiological changes that are associated with senescence. Further work is currently in progress to establish the effects of chronic dietary intervention on protein turnover in individual tissues.

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BRAIN CATECHOLAMINE CONTENT AND TURNOVER IN AGING RATS

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Abstract—The content and turnover of catecholamines were evaluated in various brain regions of young adult (4-months) and aged (24-months) male rats. Turnover was assessed from the concentrations of acid metabolites and the decline of catecholamine content after synthesis blockade with α-methyl-*p*-tyrosine. Dopamine was reduced by aging in striatum, mesolimbic areas, and hypothalamus. Dopamine metabolites and turnover rate were significantly lower in striatum and mesolimbic areas of aged than of young animals. Hypothalamic norepinephrine content and turnover rate were unchanged in aged compared to young rats but its metabolite (MHPG-SO_4) was increased in the cortex. These findings point to an extensive impairment of brain dopamine metabolism in aging rats, whereas norepinephrine seems to be less impaired.

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INTRODUCTION

AGING is associated with a progressive deterioration of CNS. Alterations in biogenic amine systems were reported in selected regions of both human and rodent aged brain (for reviews see Burchinsky, 1984; Pradhan, 1980; Rogers *et al.*, 1984; Samorajski, 1977).

Dopamine (DA) content was generally decreased in striatum (Adolfsson *et al.*, 1979; Algeri *et al.*, 1983; Bhaskaran and Radha, 1984; Carlsson and Winblad, 1976; Demarest *et al.*, 1980; Strong *et al.*, 1982) and norepinephrine (NE) in brain stem, including locus coeruleus (Ponzio *et al.*, 1978; Robinson *et al.*, 1972; Sun, 1976), but results in other brain regions (e.g., hypothalamus) were controversial (Bhaskaran and Radha, 1984; Demarest *et al.*, 1980; Finch, 1978; Ponzio *et al.*, 1978; Simpkins *et al.*, 1977; Sun, 1976).

Reports on age-related changes in catecholamine turnover are scanty and inconclusive (Algeri *et al.*, 1983; Demarest *et al.*, 1980) in spite of the fact that turnover rate, providing a dynamic measure of both content and metabolism, is much more meaningful than steady-state.

In the present study, age-related changes of catecholamine content and turnover in various rat brain regions were assayed by two procedures. The level of acid metabolites, homovanillic (HVA) and dihydroxyphenylacetic (DOPAC) acids for DA and methoxyhydroxyphenylglycol sulphate (MHPG-SO_4) for NE, is accepted as an index of their turnover. Turnover rate of catecholamines was also determined by measuring the α-methyl-*p*-tyrosine (α-MT)-induced decline of their brain levels at various times.

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MATERIALS AND METHODS

Male CD® – Crl:CD® (SD)BR rats aged 4 and 24 months and weighing 400–500 g and 700–800 g, respectively, were obtained from Charles River Italia (Calco, Como). None of them had visible tumors. They were housed at 22 ± 1°C with a 12-hour light/dark cycle (6:00 a.m. to 6:00 p.m.) and were allowed free access to water and standard diet (Charles River 4RF21 with 22% protein content).

To measure catecholamine metabolites, groups of five to eight animals were killed by decapitation. Brains were rapidly removed and dissected (Glowinski and Iversen, 1966) on ice-cold glass plates. Hypothalamus, striatum, mesolimbic areas (tuberculum olfactorium + nucleus accumbens), and cerebral cortex were taken, frozen on dry ice, and stored at −80°C until assayed.

The mean weights ± S.E.M. of these regions in 4- and 24-month-old rats were, respectivelv; hypothalamus: 56.4 ± 1.8 and 59.1 ± 3.6 mg; striatum: 117.4 ± 2.1 and 135.5 ± 3.6 mg; mesolimbic areas: 67.3 ± 2.4 and 79.2 ± 10.4 mg; cortex: 948.7 ± 14.3 and 986.2 ± 30.2 mg.

Spectrofluorometric methods were used for determining DA metabolites in striatum (HVA, DOPAC) and mesolimbic areas (DOPAC) (Westerink and Korf, 1977). NE (Westerink and Korf, 1977) was assayed in hypothalamus and MHPG-SO_4 (Meek and Neff, 1972) in cerebal cortex.

Catecholamine content and kinetics of turnover were determined by assaying DA and NE before and after inhibiting their synthesis with α-MT (Brodie *et al.*, 1966). Groups of six to eight rats were injected with α-MT (250 mg/Kg i.p.) and killed by decapitation immediately and after 1, 2, 4, and 6 hours. Striatum, mesolimbic areas, and hypothalamus were dissected and stored as described. They were extracted with 0.1 *M* perchloric acid followed by alumina absorption.

Concentrations of NE and DA were determined by HPLC with electrochemical detection (Eriksson and Persson, 1982). Turnover rate was calculated according to Brodie *et al.* (1966) and Widerlöv and Lewander (1978). The significance of differences between groups was established by Student's *t* test. Linear regression was analyzed by the least squares method.

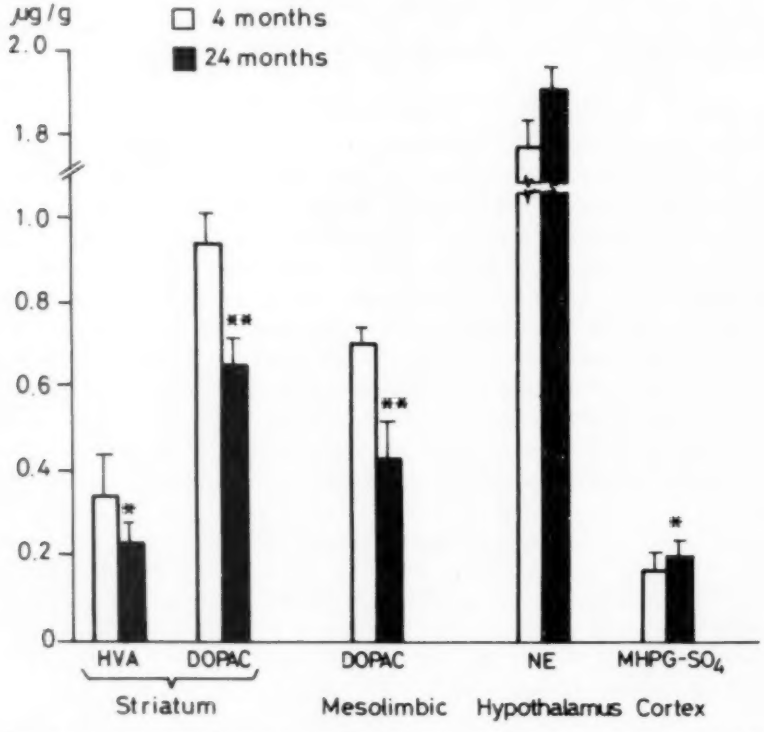

FIG. 1. Concentration of catecholamine metabolites in various brain regions of 4-months and 24-months rats. Each column represents the mean ± S.E.M. of five to eight animals.

*$P < 0.05$; **$P < 0.01$ (Student's *t* test)

RESULTS

Figure 1 shows that DA metabolites were significantly lower in striatum (HVA and DOPAC down 32%) and mesolimbic areas (DOPAC down 39%) of old rats than of young adult rats. No significant age differences were observed in hypothalamic NE. In contrast, MHPG-SO_4, the main NE metabolite in brain, was 16% higher in the cortex of old rats than of young adult animals.

As shown in Figure 2 and Table 1, the concentration of DA significantly decreased with age in mesolimbic areas (26.6%), striatum (−13%), and hypothalamus (−26%). Its turnover rate, measured by the decline of the amine after inhibition of synthesis with α-MT, was significantly slowed in mesolimbic areas (−24%) and striatum (−18%), but not in hypothalamus. The content and turnover of NE were not altered by aging in hypothalamus but increased in mesolimbic areas (+22%), though this increase was not statistically significant.

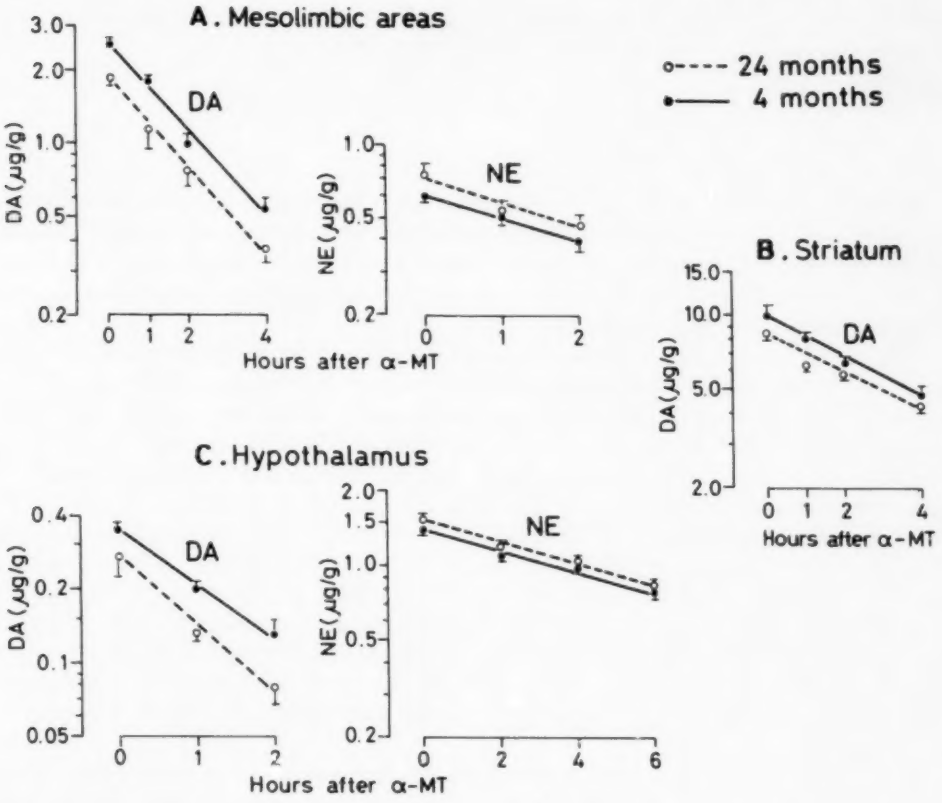

FIG. 2. Decline of catecholamine levels in mesolimbic areas (A), striatum (B), and hypothalamus (C) after injection with α-MT (250 mg/kg i.p.) in young adult (4-months ●——) and old (24-months ○----) rats. Animals were killed immediately (0 time) or 1, 2, 4, and 6 hours after α-MT. Points represent mean ± S.E.M. of six to eight animals.

Regression lines were calculated by the least squares method.

TABLE 1. CONTENT AND TURNOVER OF CATECHOLAMINES IN BRAIN AREAS OF YOUNG ADULT AND OLD RATS

Brain region	*Age (mos)*	*Amine*	*Content ng/g ± S.E.M.*	*Turnover rate μg/g/h*
Mesolimbic areas	4	DA	2518.3 ± 145.2	0.987
	24	DA	1861.3 ± 129.4**	0.752*
	4	NE	607.2 ± 34.4	0.136
	24	NE	746.5 ± 83.2	0.166
Striatum	4	DA	10039.1 ± 589.4	1.81
	24	DA	8760.6 ± 152.8*	1.49*
Hypothalamus	4	DA	359.1 ± 24.6	0.178
	24	DA	267.7 ± 41.5*	0.167
	4	NE	1393.1 ± 33.9	0.138
	24	NE	1566.5 ± 94.6	0.146

Experimental conditions are described in Figure 1. Content is the basal catecholamine value. Turnover rate is the average amount of catecholamine produced per wet weight of brain tissue per unit of time and is calculated according to Brodie et al. (1966) and Widerlöv and Levander (1978).
*$P < 0.05$; **$P < 0.01$.

DISCUSSION

A reduction in DA basal level in DA-rich areas, (striatum, mesolimbic) was accompanied in our study by a decrease in the functional state of its neurons as shown by reduced HVA and DOPAC concentrations and DA turnover rate. These results agree with those of Algeri *et al.* (1983) but not with Demarest *et al.* (1980), who both assayed DA turnover from changes in their acid metabolites.

Striatal DA synthesis from tyrosine is also decreased in aged rats (Ponzio *et al.*, 1978) in connection with reduced tyrosine hydroxylase activity (McGreer and McGeer, 1978). Levels of DA and its metabolites were repeatedly found to decrease with age in human DA-rich areas (Adolfsson *et al.*, 1979; Carlsson and Winblad, 1976; Gottfries, 1981).

These results might reflect the well-known age-related neuronal loss, loss of synapses, and/or biochemical deficits. Among the latter, a basic alteration such as impairment of the energy metabolism (Benzi *et al.*, 1980) is of paramount importance because it could account for the decrease of macromolecular biosynthesis (Dwyer *et al.*, 1980) and synaptosomal uptake (Finch, 1978; Jonec and Finch, 1975; Sun, 1976).

It is noteworthy that age affects mesolimbic DA systems at least to the same extent as those in striatum. Mesolimbic DA is involved in the control of some behaviors, including arousal, locomotion, self-administration of drugs, and cognitive functions (Costall *et al.*, 1984; Fantin and Bottecchia, 1984; Fink and Smith, 1980; Pettit *et al.*, 1984; Snowden *et al.*, 1982).

The fact that DA turnover was unchanged in aged hypothalamus is in accordance with some reports (Demarest *et al.*, 1980; Finch, 1978), but not others (Algeri *et al.*, 1983; Ponzio *et al.*, 1978). In this case variability could be related to differences in the various hypothalamic regions assayed.

In contrast to DA, NE content and turnover were not altered by aging in hypothalamus and even increased in mesolimbic areas. Cortical MHPG-SO_4 was also increased. A

tendency for this metabolite to rise had been already described in the brain of aged rats (Algeri *et al.*, 1983) and in patients with senile dementia Alzheimer type, SDAT (Gottfries, 1981).

These findings confirm the overall impression emerging from the literature that NE systems are less affected than DA by aging (McGeer and McGeer, 1978).

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UREA SYNTHESIS IN HEPATOCYTES ISOLATED FROM YOUNG AND OLD RATS

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Abstract—Effects of glucagon and epinephrine stimulation on urea synthesis from lactate, pyruvate, NH_4Cl, and ornithine in isolated rat hepatocytes from young and old rats were compared. It was found that the production of urea from those substrates was almost the same in both age groups. The concentrations of hormones that caused the maximum effect were the same for both groups of rats. The effectiveness of the stimulation of urea synthesis by glucagon and epinephrine was the same in hepatocytes isolated from young and old rats.

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THE PROCESS of aging diminishes the capability of an organism to adapt to different changes of its internal and external environment (Comfort, 1979). Our previous experiments showed that the gluconeogenic response of hepatocytes isolated from old rats was markedly reduced when they were stimulated by glucagon but not by catecholamines (Kmieć and Myśliwski, 1983). The mammalian liver also plays an important role in the organism's protein degradation because of its unique ability to synthesize urea, which is a final product in the metabolism of amino groups of amino acids. The process of ureagenesis is controlled by the protein content of the diet and by hormones—glucocorticoids, glucagon, and catecholamines (Cohen, 1981).

Because the process of gluconeogenesis in mammalian liver shares some characteristics with the process of ureagenesis, we decided to investigate the efficiency of urea synthesis in hepatocytes isolated from young and old rats in basal conditions and under hormonal stimulation.

MATERIALS AND METHODS

Animals

Male Wistar rats (W/Bog strain) 6–8 and 22–26 months old ("young" and "old," respectively) were used. This strain of rats has been used in our laboratory for several years. The mean lifespan of these rats is 29 months, the maximal lifespan is approximately 40 months, the survivalship in the colony at 6, 12, 18, 21, 24, 27, 30, 33, and 36 months is 100, 100, 100, 91, 84, 75, 50, 20, and 7%, respectively. The rats used in these experiments showed neither loss of body weight, which could be an indication of pathological changes, nor any gross observable pathological changes. The animals were maintained on a commercial stock diet throughout their lifespan. They were fasted for 24 hours before the beginning of hepatocyte isolation, which always took place between 9:00 and 11:00 a.m.

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Isolation of hepatocytes, incubation procedure, and analytical methods

The procedure of hepatocytes isolation as used in our experiments has been described before (Kmieć and Myśliwski, 1983). The animals were anesthetised by an intraperitoneal injection of thiobutabarbital natrium (Inactin^{R}, Byk-Gulden) 100 mg/kg body weight. The liver was preperfused with Ca^{++}-free Hanks' solution, and after placing it in the perfusion apparatus the step of recirculation perfusion of 0.05% collagenase in 5 m*M* Ca^{++}-Hanks' buffer followed. After washing and purification of isolated hepatocytes, the cells (2–4 × 10^6 cells/ml) were placed in siliconized glass vessels containing 1ml of Krebs-Ringer-bicarbonate buffer pH 7.4 (Umbreit *et al.*, 1957) and/or substrates (lactate, pyruvate, NH_4Cl, alanine, ornithine) and/or hormones (glucagon or epinephrine) in concentrations indicated. The vessels were gassed with 95% O_2 and 5% CO_2 for 1 min, tightly stopped with rubber stoppers and put in a metabolic water bath set at 37°C with shaking (90 oscillations a min) – this moment is defined as the beginning of the incubation. At time intervals indicated (20, 40, or 60 min) 0.25 ml samples of cell suspensions were taken from each vessel and deproteinised by mixing with 0.1 ml of 10% (v/v) perchloric acid. After centrifugation the supernatant was neutralised with 1N KOH and after the removal of precipitate by centrifugation it was immediately used for assays.

Urea was determined enzymatically by the method of Chaney and Marbach, 1962. A calibration curve was obtained for every set of measurements.

Cell protein content in 0.25 ml of sample taken from each incubation vessel at the end of incubation was determined by the biuret method (Gornall *et al.*, 1949).

The viability of isolated hepatocytes was checked by the trypan blue exclusion test. Only cell preparations with viability of 85% or more were used. There were no differences among cell preparations from rats of different ages. The cell yield from livers of old rats was about 20% lower than that from young ones. The ultrastructure of hepatocytes from old rats didn't differ from young ones except for the presence of a greater number of "dense bodies" (Kmieć and Myśliwski, 1983).

Materials

Phenoxybenzamine was a gift of Smith, Kline, and French (Sunnyvale, CA). The following reagents were purchased: *L*-alanine, *L*-ornithine, glucagon – Sigma (St. Louis, MO), *Na*-lactate, *Na*-pyruvate – Merck, epinephrine – Koch-Light, collagenase – Boehringer (Houston, TX), urease – Biochem. Lab. University Wrocław (Poland). All other chemicals were of reagent grade and were supplied through POCh Gliwice (Poland).

Statistics

The results of experiments were estimated statistically with the aid of linear regression analysis and *t* test.

TABLE 1. UREA SYNTHESIS FROM VARIOUS SUBSTRATES IN ISOLATED HEPATOCYTES FROM YOUNG AND OLD RATS

	nmole urea/40 min/mg protein	
Substrates	*Young rats (6–8 mo.)*	*Old rats (22–26 mo.)*
Control – without substrates added	8.6 ± 1.6 (21)	9.5 ± 1.9 (24)
Lactate + pyruvate (9:1 m*M*)	10.2 ± 1.7 (7)	11.4 ± 2.3 (9)
Alanine 10 m*M*	24.3 ± 1.7 (7)	27.1 ± 5.1 (9)
NH_4Cl 10 m*M*	18.7 ± 2.8 (8)	17.2 ± 3.5 (9)
NH_4Cl (10m*M*) + ornithine (2 m*M*)	59.5 ± 8.9 (8)	54.2 ± 7.8 (8)
Lactate + pyruvate + NH_4Cl + ornithine (9:1:10:2 m*M*)	93.2 ± 14.4 (9)	97.8 ± 15.3 (9)

Hepatocytes were incubated for 40 min as described in Methods. Endogenous urea synthesis was subtracted from values of urea production from exogenous substrates. The results are mean ± SEM from the number of experiments given in parenthesis.

RESULTS AND DISCUSSION

The values of urea production from endogenous and exogenous substrates in hepatocytes isolated from young and old rats are presented in Table 1. The compounds used as substrates and their concentrations were established on the basis of the earlier works of Krebs *et al.*, 1976. In both animal groups, lactate and pyruvate, alanine or ammonia ions provided relatively low rates of urea synthesis, whereas the addition of ornithine resulted in a substantial increase of ureagenesis. The highest rate of urea production was achieved when a mixture of lactate, pyruvate, ammonia ions, and ornithine was used. These results clearly demonstrate a lack of any age-dependent difference in the rate of urea synthesis from endo- and exogenous substrates in rat liver parenchymal cells.

The effects of glucagon and epinephrine on ureagenesis in hepatocytes from young and old rats are shown in Table 2. In both age groups the greatest stimulation of urea production was observed with glucagon at the concentration of $5 \times 10^{-5}M$ and with epinephrine at the concentration of $10^{-5}M$. Both hormones stimulated urea synthesis in hepatocytes from young and old rats to the same extent. Because the action of epinephrine was almost completely abolished in the presence of an alpha-adrenergic antagonist phenoxybenzamine, it was possible to conclude that the activation of ureagenesis by epinephrine was due to its interaction with alpha-adrenergic receptors in hepatocyte plasma membrane. The urea synthesis in isolated hepatocytes was linear up to 1 hour both in the absence and in the presence of hormones (Figures 1a. and 1b.).

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TABLE 2. EFFECTS OF GLUCAGON AND EPINEPHRINE ON UREA SYNTHESIS FROM EXOGENOUS SUBSTRATES IN ISOLATED HEPATOCYTES FROM YOUNG AND OLD RATS

Hormones	*nmoles urea/40 min mg protein*		*% of control value*	
	Young rats (6–8 mo.)	*Old rats (22–26 mo.)*	*Young rats*	*Old rats*
Control—without hormones added	101.5 ± 15.9 (25)	107.4 ± 17.3 (22)	100	100
Glucagon				
$5 \times 10^{-6}M$	144.1 ± 18.8[a](8)	149.2 ± 20.7[a](9)	142	138
$5 \times 10^{-7}M$	143.0 ± 19.2[a](8)	151.4 ± 24.3[a](7)	141	141
$2.5 \times 10^{-8}M$	107.4 ± 16.1 (7)	108.5 ± 22.4 (9)	106	101
Epinephrine				
$10^{-5}M$	137.3 ± 25.7[a](8)	143.6 ± 21.7[a](9)	135	133
$10^{-6}M$	105.6 ± 25.7[a](7)	106.1 ± 18.9 (8)	104	99
Epinephrine $10^{-5}M$ + PBX $10^{-4}M$	105.0 ± 19.3 (5)	110.3 ± 18.0 (6)	104	102
PBX $10^{-4}M$	103.3 ± 16.7 (4)	105.2 ± 17.8 (4)	102	98

Hepatocytes were incubated for 40 min with the mixture of substrates:lactate, pyruvate, NH_4Cl, and ornithine (9:1:10:2 mM, respectively). The results are mean ± SEM from the number of experiments given in parenthesis.

PBX = phenoxybenzamine

[a]—difference against the control value is statistically significant, $P < 0.05$.

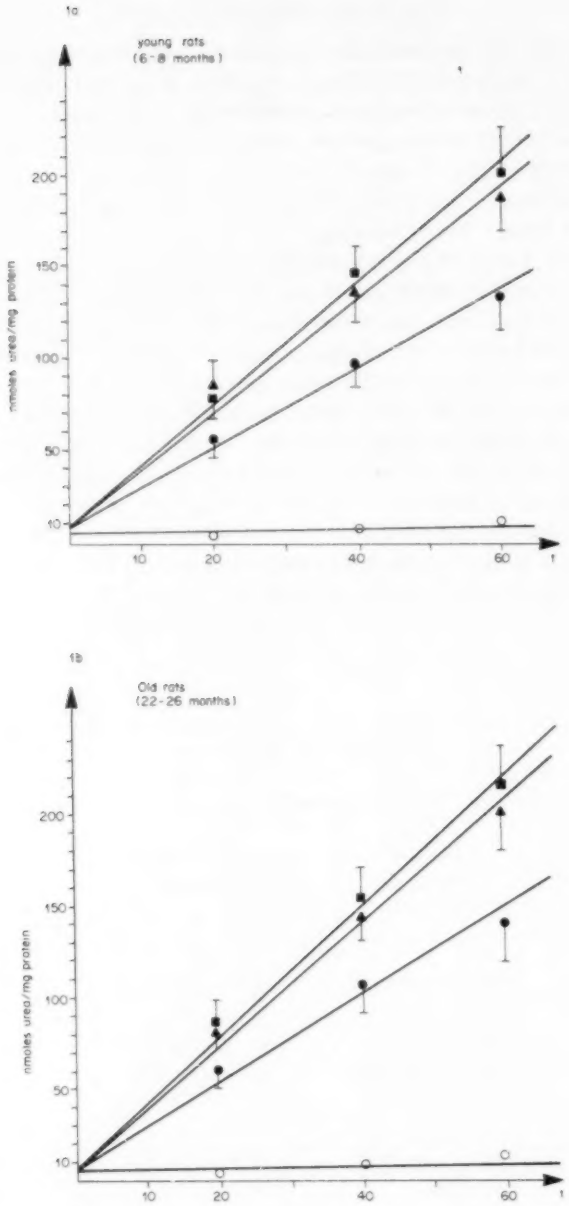

FIG. 1a. and 1b. Time dependence and the effects of glucagon and epinephrine on urea synthesis from lactate, pyruvate, NH_4Cl, and ornithine (9:1:10:2 mM, respectively) in isolated hepatocytes from young (Fig. 1a.) and old (Fig. 1b.) rats. Open circles—control, no substrates, and hormones added, closed circles—only substrates present, triangles—substrates and epinephrine $10^{-5}M$, squares—substrates and glucagon $5 \times 10^{-6}M$. Each point represents the mean of six experiments, vertical bars represent SEM. Abscissa—time of incubation, ordinate—synthesis of urea. The differences between values of urea production in the presence and in the absence of hormones were statistically significant for every period of incubation in both age groups, $P < 0.05$.

The common characteristics that ureagenesis share with gluconeogenesis include joint intermediary reactants (such as £-ketoglutarate and aspartate), competition of these two processes for ATP (Krebs *et al.*, 1976), and activation during fasting and by hormonal stimulation (Bryła *et al.*, 1978; Hensgens *et al.*, 1980; Titheradge and Haynes, 1980). Our previous (Kmieć and Myśliwski, 1983) and present results indicate that under basal conditions and after stimulation by epinephrine the rates of glucose and urea production in isolated hepatocytes from old rats did not differ from those found in young ones. On the other hand, glucagon stimulation of gluconeogenesis but not of ureagenesis was less effective in liver cells from old rats. Searching for an explanation of this difference in the influence of aging on glucagon stimulation of both processes, one must consider the ways by which glucagon enhances glucose and urea synthesis in hepatocytes. Some authors proposed that this stimulatory effect of glucagon might be due to an increase of intramitochondrial ATP levels and of ATP/ADP ratio (Bryła *et al.*, 1978; Titheradge and Haynes, 1980). On the basis of our results we think that the effect(s) of aging on the glucagon action on rat hepatocytes must consist in specific mechanisms that are different for gluconeogenic and ureagenic pathways of metabolism. In relation to glucose synthesis we suggested in our previous paper that aging may diminish the activation by glucagon of a key gluconeogenic enzyme – pyruvate carboxylase (Kmieć and Myśliwski, 1983). This assumption was additionally supported by the present results – the lack of age-dependent changes in the efficiency of ureagenesis.

Taking into account the results of our experiments on hormonally stimulated glucose and urea synthesis in isolated hepatocytes from old and young rats, it seems possible to state that some important physiological processes that are known to be stimulated by hormones do not change in aging (e.g., ureagenesis), whereas others do (e.g., gluconeogenesis).

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EFFECT OF AGE ON RAT LIVER HEME AND DRUG METABOLISM

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Abstract—Old (24-months) rats have lower activities of hepatic δ-aminolevulinic synthase and the microsomal cytochrome P-450 monooxygenase activities—aminopyrine N-demethylase and aniline hydroxylase—as compared to young (2-months) animals. In contrast, the activity of the heme degradative enzyme, heme oxygenase, is higher in the old rats. Cytochrome P-450 and microsomal heme contents were maintained in the old. When inducibility and inhibition of these enzymes were studied, the old rats responded to the same degree as the young. These results indicate that the ability of the heme synthetic and degradative enzymes to respond to decreasing cellular heme levels is not significantly altered with age. The observations that there is a lower baseline activity of ALA-synthase and good maintenance of microsomal heme and cytochrome P-450 content, in spite of elevated heme oxygenase activity in the old, suggest that, at least in the senescent rat, hepatic heme utilization and degradation are only loosely coupled to heme production. It appears, therefore, that alternate sources of heme for cytochrome P-450 are available in the old animals. Furthermore, it is suggested that the old rat has a baseline change in ALA-synthase, heme oxygenase, and cytochrome P-450 that may be overcome under the appropriate conditions.

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INTRODUCTION

IN THE liver, heme is necessary for the formation of cytochrome P-450, the enzyme system necessary for metabolism of many drugs, steroids, and environmental chemicals (Conney, 1967). The effect of aging on the cytochrome P-450 enzyme systems in both humans and experimental animals seems to show a decline in adaptability (Adelman and Freeman, 1972). It has been suggested that hepatic drug metabolizing capacity may not be uniformly affected by age and there is individual variability among humans, as well as in animals, depending on the species, strains, and sex studied (Ouslander, 1981). The present study, therefore, was undertaken in order to investigate the effect of aging on the activities and inducibility of δ-aminolevulinic acid synthase (ALA-synthese) and heme oxygenase (HO), as well as the monooxygenase drug metabolizing enzymes—aminopyrine N-demethylase and aniline hydroxylase—in male rat livers.

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MATERIALS AND METHODS

Reagents

δ-nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD), glucose-6-phosphate (G6P), cycloheximide, δ-aminolevulinic acid, hemin, and bovine serum albumin were purchased from Sigma Chemical Company (St. Louis, MO). Glucose-6-phosphate dehydrogenase was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Aminopyrine and aniline were purchased from Aldrich Chemical Company (Milwaukee, WI). Dextran 40 was from Coulter Laboratories (Hialeah, FL).

Animal treatment

Old (24-months) Sprague-Dawley rats were initially obtained from the NIA. When these rats became unavailable, we then purchased them at 14 months of age from Charles River Breeding Laboratories (Wilmington, MA) and maintained them in suspended stainless steel cages, with two animals per cage. They were fed Purina® lab chow ad lib and drank standard tap water. The room temperature was kept at 22°C with a 12-hour diurnal cycle. They were sacrificed at 24 months after a 12-hour fast. Young rats were purchased at 1 month of age and sacrificed at 2 months of age. The results with the old rats purchased from NIA were similar to those from Charles River Breeding Laboratories. However, the animals reported in this study include only those from Charles River Breeding Laboratories.

Allyisopropylacetamide (AIA), phenobarbital, and cobalt chloride dissolved in saline were administered intraperitoneally at a dose of 25 mg, 10 mg, and 0.8 mg/100 gm weight, respectively. Control animals received equivalent volume of vehicle. No difference in enzyme activities were seen between vehicle-treated and uninjected rats. The animals were sacrificed 16 hours after injection. Rats were killed by decapitation and the livers were perfused in situ with 0.9% NaCl until totally blanched. The livers were removed and homogenized in 20% of 0.05 *M* Tris-HCl, 0.25 *M* sucrose buffer (pH 7.4). Aliquots were taken for assays of ALA-synthase and ALA-dehydratase. The tissue homogenate was centrifuged at 27,000 × *g* for 20 minutes. The supernatant was centrifuged at 105,000 × *g* for 1 hour and the resulting microsomal pellet was resuspended to 0.1 *M* potassium phosphate buffer, pH 7.4, and used for determination of heme content, cytochrome P-450, amino-pyrine *N*-demethylase, aniline hydroxylase, and microsomal heme oxygenase.

ALA-synthase and ALA-dehydratase assays

The activity of mitochondrial ALA-synthase was determined in freshly prepared liver homogenate by the method described by Ibraham *et al.* (1979).

ALA-dehydratase activity was assayed colorimetrically using 50 μl of liver homogenate in the absence and in the presence of 20 m*M* dithiothreitol (Sassa *et al.*, 1974).

Aminopyrine N-demethylase assay

Aminopyrine *N*-demethylase activity in the liver microsomes was measured by the method of Alvares and Mannering (1970). The following mixture was used: NADP, 2 m*M*; semicarbazide hydrochloride, 37.5 m*M*; magnesium chloride, 20 m*M*; glucose-6-phosphate dehydrogenase, 2 units; aminopyrine, 2 m*M* dissolved in 1.15% KCl; 0.2 moles of KH_2PO_4 Na_2HPO_4 buffer, pH 7.4; 1 ml of microsomal suspension equivalent to 5 mg protein and 1.15% KCL solution to a final volume of 5 ml. Formaldehyde produced from the demethylation reaction was measured by the method of Nash (1953).

Aniline hydroxylase assay

The activity of aniline hydroxylase in the microsomal fraction was determined according to the method of Imai *et al.* (1966). The incubation mixture in a final volume of 2.5 ml contained NADP (1.5 μmoles), glucose-6-phosphate (50 μmoles), nicotinamide (100 μmoles), potassium phosphate buffer (280 μmoles), pH 7.4. These were incubated with 2–4 mg microsomal protein for 20 minutes at 37°C. The reaction was terminated by adding 1 ml of 20% trichloroacetic acid, transferred to conical centrifuge tubes and spun down at 600 × *g* for 10 minutes. Two ml of 0.5 *M* NaOH-2% phenol solution and 2 ml of 1 *M* Na_2CO_3 solution were added to 2 ml of the supernatant and absorbance at 650 nm was determined after 20 minutes (Ibraham *et al.*, 1981).

Cytochrome P-450

Cytochrome P-450 content was measured from the reduced CO difference spectrum by using sodium dithionite as the reducing agent (Omura and Sato, 1964). The absorbance difference between 450 and 490 nm was used to calculate the cytochrome P-450 content, using molar extinction coefficient of 91 $nM^{-1}cm^{-1}$.

Microsomal heme content

Microsomal heme was measured by the pyridine hemochromagen method (Ibraham *et al.*, 1979). The absorbance difference between 557 and 531 nm was used to calculate the heme content, using a molar extinction coefficient of 21 $nM^{-1}cm^{-1}$.

Heme oxygenase assay

Heme oxygenase activity was measured in the microsomal fraction as described previously (Ibraham *et al.*, 1979). The volume of the assay medium was 1.0 ml and consisted of 3.0 mg of the 105,000 × *g* microsomal supernatant fraction, as a source of biliverdin reductase, 0.8 m*M* NADP, 2 m*M* glucose-6-phosphate, 0.2 units of glucose-6-phosphate dehydrogenase, 5 mg microsomal protein, and 17 nmoles hemin. The reaction was terminated after 15 minutes by placing the tubes on ice and adding 0.1 ml cold H_2O. The bilirubin was extracted three times with 2 ml of $CHCl_3$. The $CHCl_3$ fraction was then evaporated under nitrogen, oxygen-free, to a final volume of 0.8 ml and the extract scanned in a Cary Model 14 spectrophotometer. The concentration of bilirubin was calculated from the difference in absorption between 456 nm and 517 nm utilizing an extinction coefficient of 58 $nM^{-1}cm^{-1}$.

Protein determination

Protein concentration was determined using bovine serum albumin (Fraction V) as a standard (Ibraham *et al.*, 1979).

RESULTS

Effects of age on ALA-synthase, ALA-dehydratase, heme oxygenase, and microsomal heme in rat liver

The basal activities of mitochondrial ALA-synthase, ALA-dehydratase, and microsomal heme oxygenase from young and aged rats were compared. It is clear (Table 1) that the activity of ALA-synthase is decreased in 24-month-old rats as compared to those 2-months old ($P < 0.01$), in agreement with other investigators using rat brain, heart, and liver (Paterniti *et al.*, 1978). ALA-dehydratase, the second enzyme in heme biosynthesis, was not changed in the liver of young or old rats. The enzyme activity was assayed in the absence and presence of dithiothreitol, a known stimulatory agent for enzyme activity in red cells (Sassa *et al.*, 1974). Dithiothreitol has no effect on ALA-dehydratase in both young and old rats' livers.

Heme oxygenase, the rate-limiting enzyme in heme degradation, was increased in aged animals ($P < 0.01$). As can be seen in Table 1, in spite of a lower ALA-synthase activity, the total microsomal heme was not significantly lowered in aged rats. It must be assumed,

TABLE 1. AGE-RELATED CHANGES IN HEPATIC CELLULAR HEME ENZYMES

Age of animals	*ALA-synthase nmoles ALA formed/g liver/hr*	*ALA-dehydratase nmoles PGB formed/ mg/min*		*Heme oxygenase pmoles bilirubin formed/10 mg/min*	*Microsomal heme nmoles heme/mg*
		−DDT	+DTT		
2 months	24.22 ± 3.82	7.82 ± 0.30	8.73 ± 0.35	280 ± 41	1.24 ± 0.11
24 months	16.31 ± 3.12*	8.10 ± 0.28	8.91 ± 0.45	395 ± 41*	1.09 ± 0.14

Note: Each value represents the average of six experiments in which two livers of young rats were pooled in each case ± SEM.

*Differs from young with $P < 0.01$ by Student's *t*-test analysis.

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therefore, that an adaptive mechanism develops to compensate for the low basal level of ALA-synthase activity (see Discussion).

Effect of inducers on rat liver ALA-synthase, ALA-dehydratase, heme oxygenase, and microsomal heme

In order to determine whether these age-dependent changes were reversible, animals were injected with various known inducers. Allylisopropylacetamide (AIA) degrades intracellular heme to porphyrin-like "green pigments" (not biliverdin) (DeMatteis, 1978). Treatment with this compound (Table 2) resulted in a significant decrease in microsomal heme concentration and in 6-fold induction of ALA-synthase activity in both young and old animals ($P < 0.01$). There was no signficant change in either ALA-dehydratase or heme oxygenase. Thus, it appears that as the regulatory heme pool falls, ALA-synthase is released from feedback repression.

When animals were given phenobarbital, which induces cytochrome P-450 production and utilization of the regulatory heme pool (DeMatteis and Gibbs, 1972), there was a 2.4-fold increase in ALA-synthase activity in both young and old rats. There was also a significant increase in ALA-dehydratase activity. There was no significant change in heme oxygenase but there was an increase in microsomal heme concentration. The dose of phenobarital was chosen as it did not result in the death of the old animals.

Rats were then given cobalt chloride, which is known to be an inducer of heme oxygenase (Maines and Kappas, 1975). However, a dose was chosen that did not induce the enzyme in young rats (0.8 mg/100 gm body weight). As can be seen in Table 2, the old rats responded with a 4-fold increase in heme oxygenase activity and a reciprocal decrease in microsomal heme ($P < 0.01$). Of note, however, is that $CoCl_2$ treatment resulted in a significant decrease in ALA-synthase and ALA-dehydratase activity rather than an increase ($P < 0.01$).

TABLE 2. EFFECT OF AIA, PB, AND CO ON HEME SYNTHETIC AND DEGRADATIVE PATHWAYS

Assay	*Age of animals*	*Untreated animals*	*AIA*	*PB*	*CO*
ALA-synthase	2 months	24.22 ± 3.82	144.51 ± 9.64	57.33 ± 5.32	26.63 ± 3.12
nmoles ALA formed/g liver/hr	24 months	16.31 ± 3.12*	95.24 ± 6.83*	40.82 ± 4.64*	12.52 ± 3.11*
ALA-dehydratase	2 months	7.82 ± 0.30	7.94 ± 0.28	10.13 ± 0.61	6.91 ± 0.44
nmoles PBG formed/mg/min	24 months	8.10 ± 0.28	9.41 ± 0.43	9.92 ± 0.52	2.43 ± 0.28*
Heme oxygenase	2 months	280 ± 41	294 ± 32	198 ± 21	211 ± 41
pmoles bilirubin formed/10mg/min	24 months	395 ± 47*	406 ± 38*	396 ± 29*	867 ± 59*
Microsomal heme	2 months	1.24 ± 0.11	0.72 ± 0.14	2.14 ± 0.23	0.92 ± 0.16
nmoles heme/mg protein	24 months	1.09 ± 0.14	0.51 ± 0.18*	1.90 ± 0.26	0.46 ± 0.13*

Note: Each value represents the average of four experiments in which two livers of young rats were pooled in each case ± SEM. The untreated controls were those used in Table 1.

*Differs from young with $P < 0.01$ by Student's *t*-test analysis.

Hemin inhibition of AIA induction of ALA-synthase

Hemin (0.8 mg/100 gm body weight) was administered intraperitoneally to young and old rats 6 hours after AIA injection. Inhibition of ALA-synthase induction in both young and old animals was very significant 3 hours after hemin administration ($P < 0.01$) (Figure 1). Similar effects were observed when rats were given cycloheximide (1.8 mg/100 gm body weight). The enzyme decrease was observed in both young and old animals.

Effect of age on cytochrome P-450 and its monoxygenase activities

The effect of age on cytochrome P-450 is shown in Table 3. There was a (19%) decrease in cytochrome P-450 content, which did not reach statistical significance. However, aminopyrine *N*-demethylase and aniline hydroxylase activities were significantly reduced, 43% and 28%, respectively, in old rats ($P < 0.01$).

Effect of AIA, phenobarbital, and $CoCl_2$ on cytochrome P-450 and monooxygenase activities

As shown in Table 3, the ability to respond to AIA, phenobarbital, and $CoCl_2$ is not lost with senescence. When rats were given AIA, which is known to degrade cytochrome P-450, there were similar decreases in cytochrome P-450 content and monooxygenase activities regardless of age ($P < 0.01$). When the animals were given phenobarbital, a

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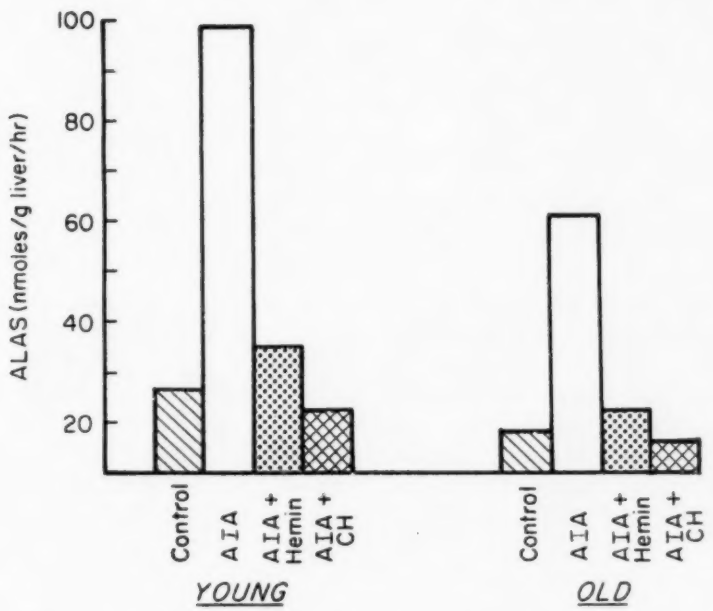

FIG. 1. Hemin and cycloheximide inhibition of AIA induction of ALA-synthase in young and old rats: Hemin (0.8 mg/100 gm body weight) or saline were administered intraperitoneally to young and old rats 6 hours after AIA injection. After 3 hours, the rats were sacrificed and ALA-synthase activity was determined. Values represented are the average of two rats.

TABLE 3. RESPONSE OF CYTOCHROME P-450 MONOOXYGENASE ACTIVITIES IN 2- AND 24-MONTH-OLD RATS: EFFECTS OF AIA, PB, AND CO

Assay	*Age of animals*	*Untreated animals*	*AIA*	*PB*	*CO*
Cytochrome P-450	2 months	0.85 ± 0.09	0.43 ± 0.08	1.30 ± 0.12	0.66 ± 0.10
nmoles/mg protein	24 months	0.69 ± 0.11	0.31 ± 0.09	1.15 ± 0.13	0.31 ± 0.08*
Aniline hydroxylase	2 months	40.43 ± 2.14	30.12 ± 3.53	66.11 ± 3.24	30.71 ± 3.42
nmoles p-aminophenol formed/mg/hour	24 months	30.64 ± 2.50*	24.94 ± 2.81	77.82 ± 4.13	12.91 ± 2.13*
Aminopyrine *N*-demethylase nmoles HCHO/mg/min	2 months	7.22 ± 0.13	4.93 ± 0.16	9.62 ± 0.36	5.42 ± 0.25
	24 months	4.14 ± 0.12*	3.23 ± 0.19	8.70 ± 0.42	1.13 ± 0.31*

Note: Rats were injected intraperitoneally with phenobarbital (PB), 10 mg/100 gm body weight. Allylisopropylacetamide (AIA) and cobalt chloride (CO) were injected subcutaneously at a dose of 15 and 0.8 mg/100 gm body weight, respectively. Rats were starved and sacrificed by decapitation 12 hours later. In the untreated rats, each value represents the average of seven experiments in which two livers of young rats were pooled in each case. In the treated animals, each value represents the average of four experiments performed in triplicate.

*Differs from young with $P < 0.01$ by Student's *t*-test analysis.

known inducer of cytochrome P-450 and the monooxygenase system, there was also a similar increase in these functions ($P < 0.01$). On the other hand, when the rats were given $CoCl_2$, the young rats showed a 22% decrease in cytochrome P-450 content ($P < 0.01$). In the old animals, cytochrome P-450 was decreased by 55% ($P < 0.01$). Aniline hydroxylase and aminopyrine *N*-demethylase activities were affected by the cobalt injection in the old animals, showing a 59% and 76% decrease in activities ($P < 0.01$), whereas in the young rats, they were down by 24% and 22%, respectively ($P < 0.01$). This decrease in cytochrome P-450 content and activities occurred at a time that heme oxygenase activity was stimulated by cobalt in the old rats.

DISCUSSION

Previous investigators have reported an age-related decrease in hepatic ALA-synthase activity in rat liver, heart, and brain (Paterniti *et al.*, 1978). In the present study, we have confirmed the decrease in baseline ALA-synthase in the livers of old rats. However, when inducibility of this enzyme was studied, we found that the older animals responded similarly to the young ones. Treatment of the animals with the known porphyrinogenic compound, AIA, resulted in a 6-fold increase in ALA-synthase in both old and young animals. It appears, therefore, that as AIA degrades intracellular heme to "green pigments" (DeMatteis, 1978), there is relief of heme-induced feedback repression of ALA-synthase regardless of age. This was confirmed by treating the animals with phenobarbital, an inducer of cytochrome P-450 content. Phenobarbital has been thought to accelerate heme utilization by the newly formed cytochrome P-450 and thereby, decrease the intracellular heme pool (DeMatteis and Gibbs, 1972). It does not significantly affect heme degradation.

When we injected rats with $CoCl_2$ (0.8 mg/100 gm body weight) (Ibraham *et al.*, 1981), the older rats responded with a 4-fold increase in heme oxygenase activity and a reciprocal decrease in microsomal heme. ALA-synthase activity, however, did not increase as would be expected with relief of heme-induced feedback repression. This may be explained by

the previously reported inhibition of ALA-synthase by $CoCl_2$ (Ibraham *et al.*, 1981). The mechanism by which hemin inhibited ALA-synthase activity in both groups of rats is not clear. It is known that repletion of the regulatory heme pool by heme administration may inhibit the function of mitochondrial ALA-synthase (Marcus *et al.*, 1980), repress ALA-synthase mRNA formation, or inhibit the translocation of ALA-synthase from the cytosol to the mitochondria where it is activated (Kikuchi and Hayashi, 1981).

Induction of ALA-synthase in young and old animals by AIA was similarly suppressed by cycloheximide. This suggests that the induction of ALA-synthase results from *de novo* synthesis of new enzyme. The finding that with advancing age inducibility of the enzyme remained intact suggests that the control mechanisms of enzyme induction and the capacity of the mitochondria to accommodate such changes do not decrease with age. What remains striking is that the baseline activity of ALA-synthase is decreased in the older animal and that even with maximal induction, the level of activity remains lower in the older animal. The increase in young rats was 120 nmoles/g/hr, where in old rats treated with AIA, the increase was only 80 nmoles/g/hr. It may be postulated that *de novo* synthesis of ALA-synthase in older rat livers is only two-thirds of that in young rat livers as reported (Scotto *et al.*, 1983). In old rats, baseline heme oxygenase activity was found to be markedly increased in agreement with the findings of Paterniti *et al.* (1978). In spite of this and the decrease in ALA-synthase activity, there was little change in microsomal heme or cytochrome P-450 content. It appears that heme catabolism via heme oxygenase does not decrease with age; indeed, it seems to be increased. That heme levels remain fairly constant in the old animals suggests that heme utilization and degradation is only loosely coupled to heme production in rat liver. It is possible that the hepatic heme levels reflect the transport of heme from one tissue to another. For example, Muller-Eberhard and Liem (1974) have suggested that liver cells take up heme from circulating hemopexin. Correia *et al.* (1979) have presented evidence that exogenously supplied heme equilibrates with an unknown heme pool which provides heme for cytochrome P-450 formation. Paterniti *et al.* (1980) have also suggested that the regulation of the heme pool used for the synthesis of various intracellular hemoproteins is complex and is not simply related to endogenous hepatic synthesis and degradation.

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It is clear that age affects the various heme synthetic and degradative enzymes differently. ALA-synthase seems to be at a lower level in the old rats and could conceivably be due to alternate sources of hepatic heme or a decrease in protein synthesis. Another consideration to explain this, however, might be due to an altered enzyme with reduced catalytic capacity. In addition, altered hormonal states, nutritional balance, or the presence of unidentified environmental factors all could affect ALA-synthase. ALA-dehydratase, in contrast, did not seem to be altered in the old animals.

Heme oxygenase activity was increased in the older animals and furthermore, was more sensitive to the presence of low concentrations of $CoCl_2$. Again the differences in this enzyme activity could be explained in several ways, including a higher concentration of heavy metals with age, such as iron (Freedman and Marcus, 1980).

When cytochrome P-450 was examined as a function of age, it was clear that although there was only a slight decrease in content, the old rats had a marked decrease in the aminopyrine-*N*-demethylase and aniline hydroxylase activities. These results may represent an impairment of electron transport with aging. This could result from a change in the microsomal membrane or it may reflect rather selective deficiencies in one of the cytochrome P-450 species, which are required for drug metabolism.

Our results with phenobarbital induction of cytochrome P-450 content and activity,

however, demonstrated that senescent rats are able to increase these functions as well as the young. This induction effect of phenobarbital cannot be explained by the rather trivial decrease in heme oxygenase activity with its resultant decrease rate of turnover. We, therefore, postulate that the senescent animals have alternate sources of heme for cytochrome P-450. Other explanations are also conceivable; for example, there could be an increase in heme utilization for cytochrome P-450 that is facilitated in some manner when heme oxygenase is elevated. Badaway (1982) has recently proposed that in mammalian liver, the regulatory heme pool is not utilized by cytochrome P-450 but may be closely associated with tryptophan pyrrolase in the cytosol. This would suggest that alternate pathways for cytochrome P-450 heme are used at any age and is consistent with work in chick embryo hepatocytes (Giger and Meyer, 1981).

In the present study we have not attempted to determine at what point in the rats' life these changes in heme and drug metabolism occur or if they are progressive throughout the life span. Our results, however, indicate that even in the aged rat the reported changes in ALA-synthase, heme oxygenase, and cytochrome P-450 may be overcome under the appropriate conditions.

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GENOTYPIC VARIATION IN AGE-RELATED CHANGES IN PERIPHERAL CATECHOLAMINES

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Abstract—The effects of aging on catecholamine concentrations in heart ventricle, aorta, and adrenal gland were evaluated in two inbred mouse strains (C57BL/6J and DBA/2J) and an F_1 hybrid ($B6D2F_1$). The effects of aging on ventricular norepinephrine (NE) levels were found to be strain specific; only the C57BL/6J strain showed a significant decline in ventricular NE levels with age. Though all strains showed a decline in ventricular epinephrine (EPI) levels with age, the magnitude of the decrease varied between strains. An age-related increase in ventricular dopamine (DA) levels was observed in each of the strains. Although strain differences in catecholamine levels were also observed in aorta and adrenal gland, no age-related changes in catecholamine concentrations were noted in any of the strains. The findings indicate strain and organ specific changes in the effects of aging on catecholamine concentrations in peripheral organs.

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SYMPATHETIC NERVOUS system control of cardiovascular function is altered during aging. The concentrations of norepinephrine (NE) and epinephrine (EPI) in heart tissue decrease with age (Frolkis *et al.*, 1970; Gey *et al.*, 1965; Martinez *et al.*, 1981). The release of NE in response to immobilization stress has been found to be delayed in aged rats (Chiueh *et al.*, 1980). Further, the response to adrenergic stimulation has been shown to be significantly diminished in aged rodents (Coutoure and Regoli, 1980; Fleish, 1971; Fleish *et al.*, 1970; Simpkins *et al.*, 1983; Tuttle, 1976). Though these findings, and observations in other peripheral organs (Reis *et al.*, 1977; Simpkins *et al.*, 1983), indicate a progressive impairment of sympathetic neurotransmission with age, there are some suggestions of genotypic differences in terms of the onset and magnitude of these changes. The decline in heart auricle NE levels reported by Martinez *et al.* (1981) was observed only in male rats; no age related changes in NE levels were detected in female rats. Fleish *et al.* (1970) reported strain differences in the onset of age related reductions in aortic β-receptor activity. Differences in age related hypertrophy of the heart also have been noted as a function of sex and breeding history (Shreiner *et al.*, 1969).

There is growing evidence that genotypic differences are a major source of interindividual variability in the effects of aging. This is most apparent in terms of the longevity differences that have been reported between inbred mouse strains (e.g., Meyers, 1978).

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Strain-specific changes with age have also been observed in brain neurotransmitter synthetic enzyme activity (e.g., Waller *et al.*, 1983), adrenal-pituitary responsivity to stress (Eleftheriou, 1975), and behavior (e.g., Ingram *et al.*, 1981). These findings underscore the important influence of genetic factors in aging and caution against restricting analysis of the effects of aging to a single genotype.

In view of the observations in adult mice of significant strain differences in peripheral sympathetic function (Kessler *et al.*, 1972; Liang and Blizard, 1978; Slater *et al.*, 1977), the basic intent of this study was to determine whether the effects of aging on catecholamine levels in peripheral organs are likewise strain dependent. To this end, NE, EPI, and dopamine (DA) levels were determined in heart ventricle, aorta, and whole adrenal glands from adult (8–10 months) and aged (24-months) mice from 2 inbred mouse strains (C57BL/6J and DBA/2J) and an F_1 hybrid cross (B6D2F_1) between the two parental lines.

MATERIALS AND METHODS

Subjects

The subjects were adult (8 to 10-month-old) and aged (24-month-old) male mice from three different inbred strains: C57BL/6J (C57), DBA/2J (DBA), and B6D2F_1. Peripheral catecholamine levels were also determined in tissue from 30-month-old C57 mice; 30-month-old mice from the two other strains were not available in our colony at the time of this study. The aged C57 and DBA were originally purchased from Jackson Labs (Bar Harbor, ME) as retired breeders at 6–8 months of age and were maintained in our colony until 24 months of age. The aged B6D2F_1 were bred in our colony and were used as breeders until 6 months of age. Adult C57, DBA and B6D2F_1 mice were obtained from Jackson Labs at 5 weeks of age and were assigned to breeding pairs until 6 months of age. Once the mice were retired from breeding, they were maintained three to five to a cage with animals of the same sex. The mice were given free access to lab chow (Purina #5001) and water. A 12–12h photoperiod was in effect; light onset was at 0700 h. The subjects had been behaviorally tested 2 weeks prior to this experiment. Behavioral testing consisted of tests of exploratory behavior and spontaneous alternation.

Procedures

The mice were killed by decapitation between 1100 and 1400 hrs; the heart ventricle, aorta, and whole adrenal glands were rapidly removed and frozen on dry ice and kept at −75°C until assayed.

Tissue assay. Tissue concentrations of norepinephrine (NE), epinephrine (EPI), and dopamine (DA) were determined by high-pressure liquid chromatography (HPLC). The samples were weighed, polytroned in 20 volumes of 0.1N PCA for 30 s, and then centrifuged at 27,700 × *g* for 10 min in a refrigerated centrifuge. Two ml of 1.5 Tris HCL (pH 8.6) and 50 mg of acid-washed alumina were added to each sample. The reaction vials were shaken for 10 min. After the alumina had settled, the supernatants were aspirated without disturbing the alumina. The alumina was washed with 1 ml of water twice and allowed to dry. One ml of deionized water was added and the alumina from each sample was transferred to a microfilter and centrifuged at 1200 × *g* for 5 min. Two hundred ml of 0.1N PCA were added to each microfilter; the samples were vortexed and centrifuged. Fifty milliliters of the eluate were injected into an HPLC system (Bioanalytical Systems Inc., West Lafayette, IN) for separation of NE, EPI, and DA on a C-18 reverse phase column. Detection was achieved using a carbon paste electrode, which was set at a potential of 0.75 V. Dihydroxybenzylamine (DHBA) was added as an internal standard to all samples to correct for any losses during the extraction and separation. The mobile phase, 0.15M Monochloro Acetic Acid (pH 3.1) containing 0.002% sodium octyl sulphate, was maintained at a flow rate of 1.5 ml/min. Ventricular and aortic values were calculated as ng/mg wet weight or as ng/mg protein. Protein levels in the heart ventricle tissue were determined by the method of Lowery *et al.* (1951). Adrenal gland concentrations are reported as ng/pair of adrenals.

Statistical analysis

The presence of age and strain differences in the concentrations of NE, EPI, and DA were evaluated by means of separate analyses of variance. Where indicated by significant main effect or interaction, subsequent comparisons between means (at $P < 0.05$) were made by way of post hoc Neuman-Keuls tests, (cf., Keppel, 1982). Though catecholamine levels were also determined for 30-month C57BL/6J mice, age of strain comparisons were restricted to our findings in 8 to 10-month and 24-month mice from each strain. The findings in 30-month-

TABLE 1. THE MEAN CONCENTRATIONS (± S.E.M.) OF NOREPINEPHRINE (NE), EPINEPHRINE (EPI), AND DOPAMINE (DA) IN HEART VENTRICLE, AORTA, AND ADRENAL GLANDS OF 8 TO 10-MONTH, 24-MONTH AND 30-MONTH-OLD MALE C57BL/6J, DBA/2J, AND B6D2F$_1$ MICE

		Heart ventricle (ng/mg)			*Aorta (ng/mg)*	*Adrenal gland (ng/pair of adrenal glands)*		
Strain	*Age (mo)*	*NE*	*EPI*	*DA*	*NE*	*NE*	*EPI*	*DA*
C57BL/6J	8–10	1.225 (±0.0834) $n=11$	0.022 (±0.0030) $n=10$	0.040 (±0.0003) $n=10$	6.762 (±0.7173) $n=5$	699.4 (±100.89) $n=6$	2571.6 (±455.56) $n=7$	nd*
	24	0.848 (±0.0507) $n=9$	0.017 (±0.0015) $n=8$	0.049 (±0.0038) $n=9$	9.074 (±1.9262) $n=4$	712.9 (±74.94) $n=4$	2035.1 (±219.14) $n=4$	nd*
	30	0.763 (±0.0641) $n=7$	0.024 (±0.0068) $n=7$	0.0554 (±0.0164) $n=7$	9.535 (±1.9550) $n=6$	570.3 (±80.20) $n=7$	1623.4 (±205.66) $n=7$	nd*
DBA/2J	8–10	1.412 (±0.0380) $n=10$	0.042 (±0.0067) $n=10$	0.046 (±0.0023) $n=10$	11.013 (±2.3778) $n=8$	621.0 (±61.81) $n=5$	2802.9 (±365.10) $n=5$	46.8 (±11.87) $n=5$
	24	1.301 (±0.0858) $n=8$	0.030 (±0.0044) $n=8$	0.058 (±0.0062) $n=8$	15.569 (±2.7750) $n=6$	643.0 (±54.23) $n=5$	2749.6 (±245.66) $n=5$	36.1 (±9.10) $n=5$
B6D2F$_1$	8–10	1.149 (±0.0724) $n=9$	0.028 (±0.0032) $n=9$	0.046 (±0.0027) $n=9$	6.762 (±1.0649) $n=7$	973.1 (±77.92) $n=5$	3142.7 (±292.85) $n=5$	65.0 (±7.74) $n=5$
	24	1.302 (±0.0773) $n=7$	0.024 (±0.0015) $n=7$	0.058 (±0.0040) $n=7$	5.085 (±1.0928) $n=4$	1044.4 (±53.71) $n=5$	2930.3 (±165.30) $n=5$	57.8 (±9.57) $n=5$

*Adrenal DA concentrations were not determined in C57BL/6J mice.

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old C57 mice are summarized in Table 1 for comparative purposes. The presence of missing values and the relatively small group size precluded repeated measures or multivariate analyses. Though it has been previously suggested that age differences in peripheral catecholamine be examined by means of analysis of covariance to adjust for age differences in organ and body weight (Martinez *et al.*, 1981), preliminary analyses indicated that an unambiguous interpretation of a covariance analysis would be difficult in the present experiment in view of significant age and strain differences in the slopes of the regression lines.

RESULTS

As indicated by the findings, summarized in Table 1, strain-dependent changes in peripheral catecholamine levels were noted in this study. This observation and the presence of age-related changes, however, was not general to all tissues or neurotransmitters examined.

Heart ventricle

Comparison of heart ventricle NE concentrations revealed a significant age × strain interaction ($F[2,47] = 7.241$, $P = 0.002$). As indicated in Table 1, only the C57 strain showed a significant reduction in ventricular NE levels between 8–10 months and 24

months of age. The highest level of ventricular NE was observed in the DBA strain. No significant difference in ventricular NE levels was noted between the C57 and B6D2F$_1$ strains. Analysis of NE levels per unit protein likewise revealed a significant age × strain interaction ($F[2,45] = 7.461$, $P = 0.002$), suggesting that the above noted strain-dependent changes in NE levels with age were independent of age and strain differences in tissue weight. Ventricular NE levels (ng/mg protein) in 24-month mice (6.795 ± 0.5613) were reduced relative to 8 to 10-month-old C57 mice (10.242 ± 0.7052). No significant changes in ventricular NE levels per unit protein were detected between adult (11.737 ± 0.3163) and aged (11.428 ± 0.7531) DBA mice nor between adult (10.178 ± 0.6416) and aged (11.345 ± 0.6828) B6D2F$_1$ mice.

Significant strain ($F[2,45] = 7.704$, $P = 0.002$) and age differences ($F[1,45] = 4.502$, $P = 0.037$) in ventricular EPI concentrations were observed. Epinephrine concentrations were found to be higher in the DBA mice than either B6D2F$_1$ or C57 mice. Though declines in EPI concentrations were observed for each strain between 8–10 months and 24 months of age, the magnitude of this decline was strain dependent. In contrast to the NE and EPI findings, ventricular DA levels were found to increase with age in each of the strains ($F[2,46] = 13.232$, $P = 0.001$). As shown in Table 1, no significant strain differences in ventricular DA levels were detected.

Aorta

Significant strain differences in aortic NE levels were observed ($F[2,28] = 6.622$, $P = 0.005$); DBA mice were found to have significantly higher NE levels in the aorta than either C57 or B6D2F$_1$ mice. Though NE levels appeared to increase with age in C57 and DBA mice, these changes were not found to be statistically significant.

Adrenal gland

As shown in Table 1, significant strain differences in adrenal NE levels were observed ($F[2,24] = 13.726$, $P = 0.0001$). At both ages, B6D2F$_1$ mice were found to have significantly higher adrenal NE levels than C57 or DBA mice. Though higher adrenal EPI concentrations were also observed in B6D2F$_1$ mice, the differences between the strains were not statistically reliable. No significant differences in adrenal EPI levels were detected between 8 to 10-month and 24-month-old mice from any of the strains. Adrenal DA levels were not determined in C57 mice. No significant age-related changes in adrenal DA levels were detected in DBA or B6D2F$_1$ mice. Furthermore, no significant strain differences in adrenal DA levels were observed between DBA and B6D2F$_1$ mice at either age.

Body and organ weight

Comparisons between the overall body weights of mice revealed a significant strain × age interaction ($F[2,46] = 12.937$, $P = 0.0001$). As shown in Table 2, this reflected the finding that, although significant body weight declines were observed between 8–10 months and 24 months of age DBA and B6D2F$_1$ mice, no significant age-related change in body weight was observed in C57 mice at these ages.

The mean ventricle weight for each strain by age grouping is shown in Table 2. For comparative purposes, organ weight to body weight ratios were computed. In view of the observed differences in overall body weight, an attempt was made to adjust for body weight differences in analysis of strain and age differences in ventricular weight. Preliminary analysis indicated no age or strain differences in the slopes of the regression

TABLE 2. MEAN (± S.E.M.) BODY WEIGHT (BW), VENTRICLE WEIGHT (VW), VENTRICLE WEIGHT ADJUSTED FOR BODY WEIGHT DIFFERENCES, AND VENTRICLE TO BODY WEIGHT RATIOS ARE SHOWN FOR MALE 8 TO 10-MONTH, 24-MONTH, AND 30-MONTH-OLD C57BL/6J, DBA/2J, AND B6D2F$_1$ MICE

Strain	*Age (mo)*	*BW (g)*	*VW (mg)*	*ADJ. VW (mg)*	*VW/BW*[a]
C57BL/6J	8–10	29.3 (±1.15)	1.14 (±0.042)	1.15 (±0.077)	5.9 (±1.26)
	24	31.2 (±0.54)	1.30 (±0.058)	1.31 (±0.068)	6.4 (±0.63)
	30	26.4 (±2.18)	1.26 (±0.071)	1.37 (±0.071)	6.4 (±1.47)
DBA/2J	8–10	31.7 (±0.66)	1.51 (±0.055)	1.44 (±0.053)	6.9 (±0.25)
	24	27.5 (±1.19)	1.56 (±0.052)	1.58 (±0.063)	9.1 (±1.03)
B6D2F$_1$	8–10	34.6 (±0.85)	1.46 (±0.058)	1.36 (±0.060)	6.6 (±0.23)
	24	28.0 (±2.77)	1.29 (±0.074)	1.36 (±0.067)	7.5 (±0.68)

[a]MEAN (± SEM) × 10^{-4}

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lines. The adjusted mean ventricle weight is shown in Table 2. Significant strain (F[2,44] = 23.93, P = 0.0001) and age difference (F[1,44] = 4.39, P = 0.042) were observed in terms of the adjusted ventricle weights. The adjusted ventricle weights were found to be higher in DBA mice than either B6D2F$_1$ or C57 mice. Increases in adjusted ventricle weight were observed with age in both C57 and DBA mice; no age differences in adjusted ventricle weight were observed in B6D2F$_1$ mice. During dissection, an opaque white plaque was noted covering a portion of the ventricle in both 8 to 10- and 24-month-old DBA mice. Consistent with previous finding of Clements (1956) in adult mice, this dystrophic calcification was only observed in DBA mice.

DISCUSSION

The present findings provide further evidence of genotypic differences in the effects of aging. An age-related decline in ventricular NE concentrations was found to be specific to C57BL/6J mice; no age-related changes in ventricular NE concentrations were observed in either DBA/2J or B6D2F$_1$ mice. This strain-specific change in sympathetic neurotransmitter concentrations with age, however, was noted only in terms of ventricular NE concentrations. Though strain differences in ventricular EPI and DA levels also were noted, no significant strain differences in the effects of aging on these ventricular catecholamine concentrations were observed.

In contrast to the strain-specific decreases in heart ventricular NE and EPI levels, ventricular DA levels were found to increase with age in each of the three strains. Although DA is a precursor of both NE and EPI, the present findings suggest that the DA detected in the heart ventricle is independently regulated and may function independently, as previously suggested by other investigators (e.g., Lackovic *et al.*, 1982). The observed age-related changes in NE, EPI, and DA levels indicate that these catecholamines are differentially affected by aging.

No age-related changes in NE levels in aorta or adrenal gland were detected in any of the strains nor were any age-related changes in adrenal EPI concentrations observed in this study. Adrenal DA levels were also found not to change with age in DBA and B6D2F$_1$. The age-related decline in peripheral NE levels that was noted by Martinez *et al.* (1981) likewise was restricted to heart tissue. These findings suggest that, although age-related changes post-synaptic receptor sensitivity have been observed in various peripheral organs (e.g., Simpkins *et al.*, 1983), age-related changes in peripheral catecholamine levels may be specific to heart tissue. The strain differences noted in this study and the sex differences previously reported by Martinez *et al.*(1981) indicate that the degree, and perhaps the temporal onset, of age-related changes in heart catecholamine levels are genotype specific. The factors underlying these genotypic differences, however, remain to be identified. It is unclear whether the strain-specific reductions in ventricular NE levels with age reflect age and strain differences in the synthesis and/or release of NE. It is possible that the present findings may also reflect age and strain differences in response to the stress of decapitation. McCarty (1981) noted that, relative to findings in young rats, plasma NE and EPI levels were reduced in aged rats following a brief exposure to footshock. Eleftheriu (1975) observed strain-specific changes with age in the response to mild to moderate stress. It is noted, however, that age-related reductions in NE levels were only observed in the heart ventricle; no age or strain differences in adrenal NE or EPI concentrations were found in this study. These preliminary findings encourage a more detailed genetic analysis of age-related changes in cardiovascular function.

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CONCENTRATIONS OF PROSTAGLANDINS IN PLASMA, SEMINAL VESICLES, AND OVARIES OF AGING C57BL/6NN*IA* MICE

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Abstract—Concentrations of prostaglandin E_1 (PGE_1), and prostaglandin E_2 (PGE_2) (combined in the same radioimmunoassay) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were analyzed in circulating plasma and seminal vesicles of 3- and 26 to 27-month-old males and in circulating plasma and ovaries of 3-, 6-, 14 to 18- and 26 to 30-month-old female C57BL/6NN*ia* mice. The amount of PGE declined in the plasma ($P < 0.05$) and seminal vesicles ($P < 0.02$) of aged male mice, whereas $PGF_{2\alpha}$ concentrations remained unchanged. There were no statistical differences in plasma or ovarian concentrations of PGE or $PGF_{2\alpha}$ when comparing the various age groups of female mice. It does not appear as if age-related changes in prostaglandins play a significant role in reproductive senescence.

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INTRODUCTION

PROSTAGLANDINS, A group of naturally occurring fatty acids, are considered by many researchers to be local hormones, acting to modulate and/or mediate actions of other hormone systems. Hormonal levels are altered with age in many organs or organ systems (e.g., thyroid gland, adrenal gland, female reproductive system), therefore it is important to determine whether levels of prostaglandins change, either directly or indirectly, in these same systems. Prostaglandins are known to play an important role in reproduction and may quantitatively or qualitatively change with advancing age. They may also, in part, be responsible for or be influenced by altered gonadotropin and steroid levels noted in older female mammals. A preliminary study (Parkening *et al.*, 1981a) showed a trend toward higher circulating plasma levels of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) with age, although these differences were not statistically significant. The present study is a continuation of that research examining plasma and tissue (ovaries and seminal vesicles) concentrations of prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2), which are combined in the same assay, and $PGF_{2\alpha}$ in young, middle-aged, and aged female and young and aged male C57BL/6NN*ia* mice.

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MATERIALS AND METHODS

Male (3- and 26 to 27-month-old) and female (3-, 6-, 14 to 18-and 26 to 30-month-old) C57BL/6NN*ia* mice were acquired from Charles River Laboratories (Wilmington, MA). They were housed 2–3/cage under uniform conditions of temperature (21–23°C) and fluorescent lighting (14 h light/10 h darkness). Laboratory mouse chow and water was provided ad libitum.

All of the mice were bled unanesthetized by cardiac puncture with a heparinized syringe (23 gauge needle). This procedure yields 0.5–1.0 ml of whole blood in 15–30 seconds. Plasma samples acquired in this manner, which were analyzed by radioimmunoassay for gonadotropins and prolactin, were not statistically different from plasma samples obtained by decapitating mice. The cardiac puncture procedure permits a greater quantity of blood to be collected from individual mice.

Mice were killed between 1000–1200 h. Because the oldest female mice exhibited a constant leukocytic vaginal smear, all other females were killed when they exhibited a diestrous vaginal smear.

Once blood was collected from a mouse it was killed by cervical dislocation and its ovaries or seminal vesicles were removed, weighed, and frozen on dry ice. Blood samples were kept on ice for 15–20 min until centrifugation (1000 × *g* for 15 min at 4°C) and then 0.5–1.0 ml of plasma was transferred to a prechilled tube and 0.1 ml of 1N HCl added to adjust the pH to 3.5. The samples were stored under nitrogen at −90°C until all samples were collected (within 1 week) for radioimmunoassay. The preparation and assay of the samples was conducted as previously described (Brouhard *et al.*, 1978). Briefly, the extracted prostaglandins were dissolved with 0.2 ml solvent #1 (heptane: chloroform: ethanol: glacial acetic acid – 100:100:10:2) and added to Quik-Sep columns containing Sephadex LH 20. PGA and PGB were eluted with 15 ml of solvent #1 and discarded. PGE was eluted with 10 ml of solvent #2 (heptane:chloroform:ethanol:glacial acetic acid – 100:100:20:2). $PGF_{2\alpha}$ was then eluted from the column with 10 ml of solvent #3 (heptane:chloroform:ethanol:glacial acetic acid – 100:100:25:2). The PGE fraction was evaporated under N_2 and converted to PGB for radioimmunoassay (Brouhard *et al.*, 1978) using a commercially available kit (Clinical Assays, Division of Travenol Laboratories Inc., Cambridge, MA). The radioimmunoassay does not distinguish between PGE_1 and PGE_2 and will be referred to as PGE. $PGF_{2\alpha}$ was evaporated under N_2 and assayed with another antibody also available from Clinical Assays, which is specific for this prostaglandin. Seminal vesicles and ovaries were stored under N_2 until just prior to the assay, when they were thawed, homogenized in 1.0 ml of PBS and centrifuged for 15 min at 1000 × *g* (4°C). The supernatant was removed to a prechilled tube and 0.1 ml of 1N HCl was added. The remaining extraction, centrifugation, and radioimmunoassays were identical to those described above for plasma.

The data were statistically analyzed using the Student's *t* test (unpaired analysis) for the two groups of male mice and one-way analysis of variance for comparing the four groups of female mice. A *P* value of 0.05 was selected to indicate a significant difference.

RESULTS

Plasma concentrations of PGE were significantly lower ($P < 0.05$) in aged male mice when compared with younger males (Table 1). The concentrations of PGE in the seminal

TABLE 1. CONCENTRATIONS OF PGE_1, PGE_2, AND $PGF_{2\alpha}$ IN PLASMA AND REPRODUCTIVE TISSUES OF AGING MALE AND FEMALE C57BL/6NN*ia* MICE

Sex and age of mice	*No. mice*	*Plasma pg/ml (± S.E.M.)*		*No. mice*	*Tissue pg/mg (± S.E.M.)*	
		PGE_1 and PGE_2	*$PGF_{2\alpha}$*		*PGE_1 and PGE_2*	*$PGF_{2\alpha}$*
Male					Seminal vesicles	
3-month-old	10	1,790 ± 140	720 ± 138	8	14.9 ± 2.4	10.4 ± 2.1
26 to 27-month-old	12	1,270 ± 160*	708 ± 142	8	6.6 ± 1.7**	6.8 ± 2.5
Female					Ovaries	
3-month-old	8	1,820 ± 210	704 ± 201	–	–	–
6-month-old	7	1,640 ± 301	1,111 ± 231	7	178.3 ± 41.6	26.7 ± 6.2
14 to 18-month-old	10	1,102 ± 180	1,102 ± 271	8	185.2 ± 20.3	41.5 ± 11.1
26 to 30-month-old	15	1,220 ± 130	891 ± 104	10	294.6 ± 63.1	83.6 ± 22.0

Significantly different when compared with 3-month-old mice: *$P < 0.05$, **$P < 0.02$

vesicles of these same two groups were also lower ($P < 0.02$) on a pg/mg basis in the older animals. Six of the eight aged mice, however, had hypertrophied seminal vesicles with weights ranging from 200–580 mg. Seminal vesicle weights in younger mice ranged from 20–170 mg. There were no statistical differences in $PGF_{2\alpha}$ concentrations in plasma or seminal vesicles of these same mice.

There were no statistical differences in plasma or ovarian concentrations of PGE or $PGF_{2\alpha}$ when comparing the four age groups of female mice (Table 1).

DISCUSSION

The phenomenon of seminal vesicle enlargement in older mice of a substrain of the strain used in this study has already been described (Finch and Girgis, 1974). The bilateral condition results from an increase in fluid content within the glands. There has been no evidence of abnormal or malignant growth in microscopic sections of the seminal vesicles and the measurement of DNA content has indicated a glandular hypoplasia (Finch and Girgis, 1974). It is not known whether cells in the seminal vesicles secrete more fluid or if the ejaculatory ducts become blocked with increasing age. If PGE is being synthesized at the same rate within these cells, it may simply be diluted by the secretory product. It should be noted, however, that there were no statistical differences between $PGF_{2\alpha}$ levels in these same seminal vesicles (Table 1), indicating that the synthesis of this prostaglandin is either increased or that there is no dilution effect and that real differences exist in tissue levels of PGE in the same two age groups. The ability of the liver to synthesize prostaglandins and the influence of phenobarbitone to stimulate prostaglandins are reduced with aging in rats (Morita and Murota, 1980). There were no differences in plasma concentrations of $PGF_{2\alpha}$ when comparing 3- and 26 to 27-month-old male mice. The latter data is consistent with a previous study (Parkening *et al.*, 1982a).

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PGEs are considered to be vasodilators, therefore a reduction in blood flow to the aging mouse uterus could be in part responsible for the fewer implantation sites and the increased number of fetal resorptions that have been noted in mice 12–15 months of age (Parkening *et al.*, 1978). Blood flow within the uterus of older pregnant hamsters has been shown to be impeded (Parkening and Soderwall, 1973; Rahima and Soderwall, 1978). The increase in uterine capillary permeability at the time of implantation in the rat is dependent upon PGE_2 production in the endometrium (Kennedy, 1979). Recently it was shown that the concentrations and basal release of PGE_2 in uterine tissue of 13 to 14-month-old rats were significantly less than that in uteri of 4 to 5-month-old rats (Brown *et al.*, 1984). Concentrations and basal release of $PGF_{2\alpha}$ were not different between the two groups of rats. However, when comparing the capacity of the uteri in the two groups of rats to synthesize both PGE_2 and $PGF_{2\alpha}$, it was found that prostaglandin synthesis in older rats, including those that were ovariectomized and stimulated with progesterone and estradiol, were reduced from those found in similarly treated younger rats (Brown *et al.*, 1984). The production of uterine prostaglandins can be altered in younger rats by changing the balance of estrogen and progesterone (Pakrasi *et al.*, 1983). The balance of these steroids certainly changes with advancing age in the mouse (Finch *et al.*, 1980). In the present study uterine concentrations of prostaglandins were not examined, but based on the previously mentioned knowledge it was thought that ovarian concentrations of PGE and $PGF_{2\alpha}$ would change; however, there were no statistical differences when comparing 6-, 14 to 18- and 26 to 30-month-old mice (Table 1). This is somewhat surprising, because the

number of follicles decline with age (Parkening *et al.*, 1980) and because these prostaglandins are associated with ovarian follicles and ovulation (Brown and Poyser, 1984), it might be expected that they would decline, especially in the oldest age group.

Prostaglandins within the ovary are thought to be formed predominantly in granulosa cells of preovulatory follicles (Dennefors *et al.*, 1983). Their formation increases in the presence of LH and is highest at the time of ovulation, particularly $PGF_{2\alpha}$, which is considered to be the most important in the rupture of the follicle (Armstrong, 1981). Plasma levels of LH become elevated with increasing age in the mouse (Parkening *et al.*, 1982b), however, in the present study statistically there was no corresponding increase in the ovarian concentrations of PGE and $PGF_{2\alpha}$. This is probably due to the decline in ovarian follicles with age and the variability in the number of remaining ovarian follicles found in mice of the same age group.

It does not appear from the present studies as if changes in $PGF_{2\alpha}$ are having a profound effect on reproductive senescence in male or female mice. The changes that were apparent in PGE found in the plasma and seminal vesicles of aging male mice could be an indication of their involvement in initiating changes in the reproductive system. However, it is also possible that these alterations are merely a reflection of hormonal changes occurring earlier in these animals as a result of age-related changes in the pituitary–hypothalamic complex. It is also possible that the changes in plasma PGE result from alterations in the immune system (Hayari *et al.*, 1984) or from changes in some other organ system of the body.

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CHANGES OF VASOPRESSIN- AND OXYTOCIN-IMMUNOREACTIVE NEURONS AFTER HYPOPHYSECTOMY IN YOUNG AND OLD MICE

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Abstract—Changes of magnocellular neurons after hypophysectomy were immunohistochemically studied using antisera to arginine vasopressin (AVP) and oxytocin (OXT) in young and old female mice of the C57BL/Tw strain. AVP-immunoreactive neurons in the supraoptic and paraventricular nuclei of intact 19-month-old mice showed a marked reduction in number and immunoreactivity as compared with those of intact 3-month-old mice. Age difference of OXT-immunoreactive neurons was less pronounced than that of AVP-immunoreactive neurons. After hypophysectomy, both AVP- and OXT-immunoreactive neurons showed an intense stainability 10 days after the operation regardless of ages. However, the rate of reduction in number of immunoreactive neurons after hypophysectomy was less marked in 19-month-old than 3-month-old mice.

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INTRODUCTION

AGE-RELATED impairment of water balance has been documented by a number of investigators in mammals (for review, see Turkington and Everitt, 1976). The impairment is accompanied by morphological and functional changes in the hypothalamo-neurohypophyseal system and kidney. Histological changes in the neurosecretory cells and the posterior lobe (PL) as a function of age have been reported using conventional Gomori's staining (Rodeck *et al.*, 1960; Morrison and Staroscik, 1964; Dunihue, 1965; Kawashima and Kobayashi, 1982) and immunohistochemical technique (Sladek *et al.*, 1980a, b, 1983; Watkins and Choy, 1980). Electron microscopic studies have been carried out by Davies and Fotheringham (1980, 1981), Wilkinson and Davies (1981) and Rechardt and Hervonen (1982). Davies and Fotheringham (1980) suggested that the rate of axonal transport from the magnocellular neuronal perikarya to the PL is decreased on the basis of their findings of the increase of neurosecretory granules in the supraoptic nucleus (SON) of rats. Similarly, Sladek *et al.* (1980a, b, 1983) found in monkeys and rats a significant increase in the presence of Herring bodies and a decrease in the number of fibers immunoreactive to antisera to arginine vasopressin (AVP) and AVP neurophysin. They also found a marked reduction in the number of immunoreactive magnocellular neurons, and suggested that in aged brain the availability of AVP is decreased.

The decrease in availability of AVP can be experimentally induced by total hypophysec-

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tomy or posterior-lobectomy, and polydiptic state appears in operated animals. However, the polydipsia and polyuria eventually disappear concurrently with the reorganization of the infundibular stem to a tissue resembling the PL (Stutinsky, 1951; Billenstien and Leveque, 1955; Moll and De Wied, 1962; Kawashima *et al.*, 1966; Murakami *et al.*, 1969; Raisman, 1973; Polenov *et al.*, 1974, etc.).

The age difference of the infundibular stem during the reorganization in the mouse was reported in a separate paper (Kawamoto and Kawashima, 1985). The aim of the present paper is to report the difference between young and old mice in the hypothalamic magnocellular response to hypophysectomy.

MATERIALS AND METHODS

Animals

The current experiments were carried out on the virgin female mice of the C57BL/Tw strain at 3 and 19 months of age. They were kept in a temperature-controlled room at 12-h daily photoperiods (0600–1800) and maintained on laboratory chow (CA-1, Japan Clea Inc., Tokyo) and tap water ad libitum. Hypophysectomy was performed by transaural approach (Kawamoto, 1983). After hypophysectomy, 5% glucose was given as drinking water for 2 days and thereafter it was replaced by tap water. The completeness of hypophysectomy was verified at autopsy.

Tissue preparation

Intact mice at 3 and 19 months of age were sacrificed by decapitation on the day of diestrus. In hypophysectomized mice an autopsy was performed 10 days after the operation. The brain was taken out in each mouse and fixed in Bouin's fluid. The schedule of dehydration in a graded series of ethanol and embedding in paraplast was the same for all the brains of four groups of mice. Serial sections were frontally cut at 8 μm in thickness and divided into two or three series.

Immunohistochemical procedures

Characterization of antisera to AVP and oxytocin (OXT) and the specificity of immunohistochemical reaction have been reported previously (Kawamoto, 1985). A series of sections were incubated with anti-AVP serum (1:1600) and another series with anti-OXT serum (1:100) for 24 h at 4°C. Then they were incubated with goat anti-rabbit IgG serum (1:30) and peroxidase-antiperoxidase complex (1:60), each for 30 min at room temperature. The reaction with 3,3′-diaminobenzidine in 0.05 *M* Tris-HCl buffer (pH 7.4) containing 0.004% H_2O_2 was performed for about 5 min at room temperature. Care was given to identical conditions of immunohistochemical staining for sections of four groups of mice, and to minimize variability, a single manufacturing lot of any reagent was used throughout.

Morphometric procedures

For measuring the cell body and nuclear sizes of AVP- and OXT-immunoreactive neurons in the SON and paraventricular nucleus (PVN), 50 neurons were randomly selected in each nucleus. The measurements were accomplished using an image analyzer connected with a computer (Mutoh Industry Ltd., Tokyo). Total number of AVP- and OXT-immunoreactive neurons in the principal region of the SON and PVN was calculated based on the neurons showing a nucleolus in sections at 40 μm intervals. In addition, the total number of magnocellular neurons in the SON and PVN was calculated in other series of sections with Nissl staining. Statistical analyses for the cell body and nuclear sizes of neurons were performed by Kruskal-Wallis H and Mann-Whitney U tests, and those for the total number of neurons, by Student's *t* test.

RESULTS

Total number of magnocellular neurons

In Nissl-staining preparations, the total number of magnocellular neurons in the SON was readily counted, but in the PVN, as some magnocellular neurons contained small

amounts of Nissl substance, only those neurons showing clear deposition of Nissl substance were included in the counts. There were no statistically significant differences in the total number of neurons of the SON and PVN between intact young (n = 5) and old mice (n = 4) [SON; 5826 ± 224 (SEM) vs. 5688 ± 196, PVN; 1860 ± 106 vs. 1846 ± 100].

Vasopressin-immunoreactive neurons

In the principal region of the SON, AVP-immunoreactive neurons (AVP neurons) were predominantly found ventrally in its posterior part. Although in the PVN, AVP neurons were preferentially found ventrally in its anterior part and dorsally in its posterior part. In mice at 19 months of age, AVP neurons in both the SON and PVN were stained less

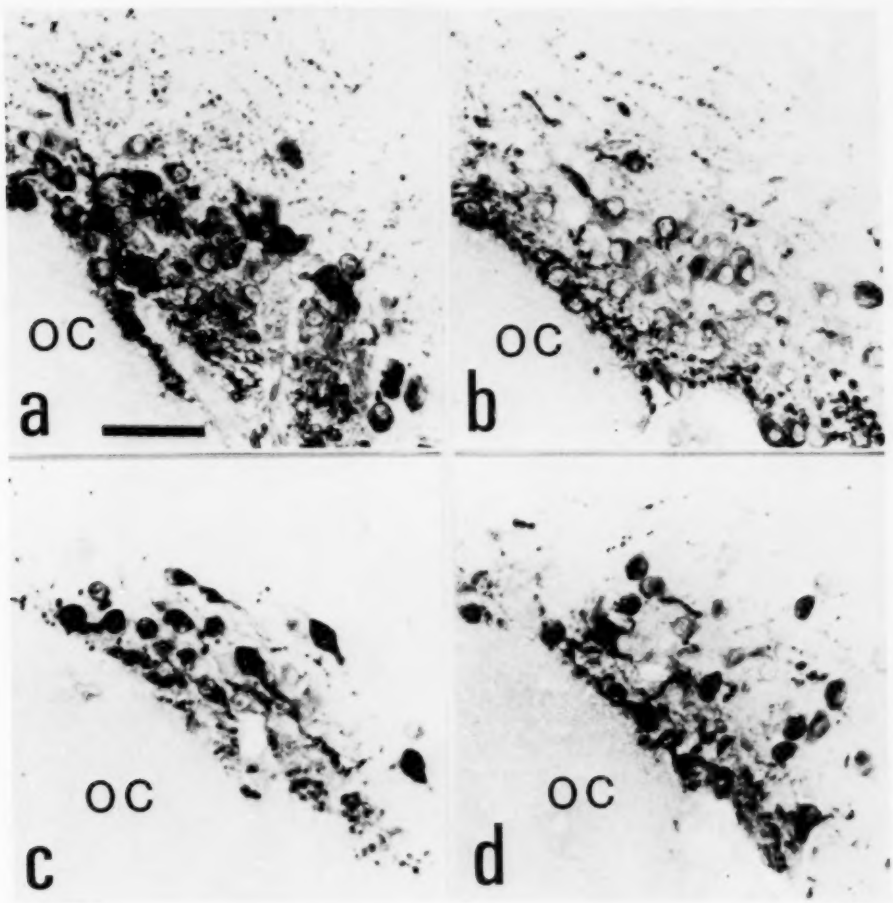

FIG. 1. Vasopressin-immunoreactive neurons in the supraoptic nucleus in young and old intact and hypophysectomized mice. OC; optic chiasma. a, c; 3-month-old mouse. b, d; 19-month-old mouse. a, b; intact. c, d; 10 days after hypophysectomy. Bar; 50 μm. The magnification is the same for the FIG. 1a.-d.

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strongly than those in 3-month-old mice (Figures 1a, b and 2a, b). The stainability to anti-AVP serum was more variable among different neurons in old mice than in young mice, being particularly evident in the SON (Figure 1a, b). At the same time, the density of AVP-immunoreactive fibers (AVP fibers) was decreased in old mice within and in the vicinity of the SON. The decrease was less extensive in the PVN than the SON (Figure 2a, b).

After hypophysectomy, some neurons in the SON and PVN showed a remarkable deposition of AVP in their perikarya, whereas some other neurons were only weakly reactive to anti-AVP serum (Figures 1c, d and 2c, d). The number of AVP neurons and AVP fibers projecting from the perikarya were decreased in hypophysectomized mice in both age groups. Table 1 shows the quantitative changes of AVP neurons in the SON and PVN 10 days after hypophysectomy in young and old mice. There were no differences

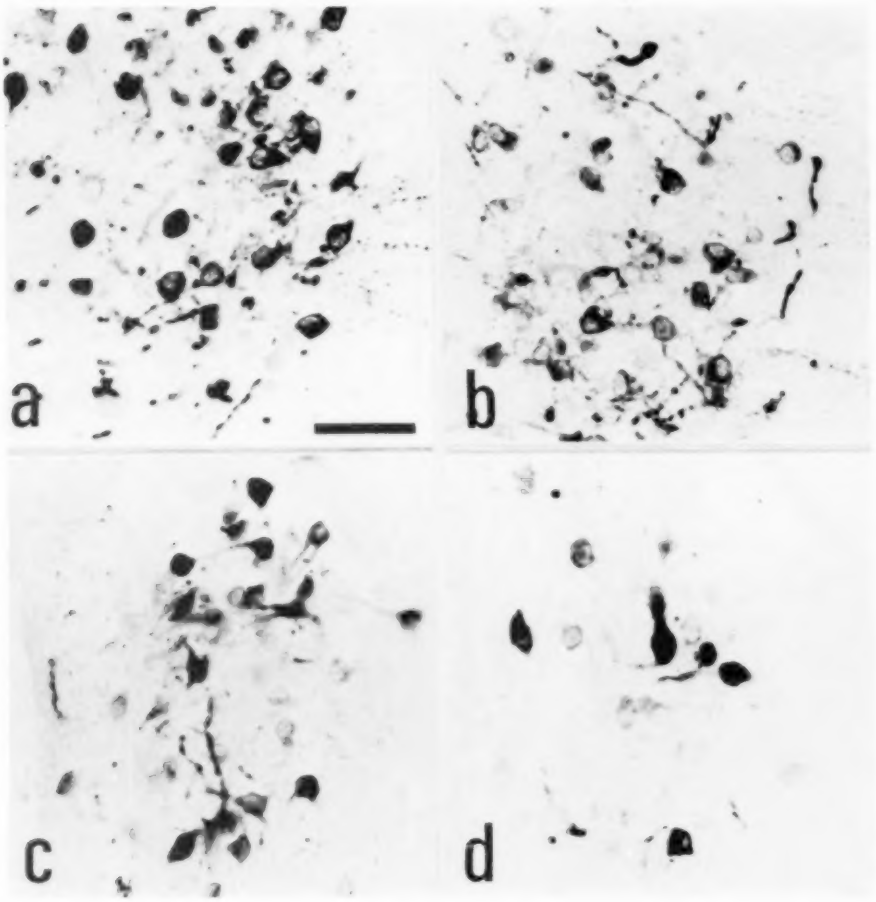

FIG. 2. Vasopressin-immunoreactive neurons in the paraventricular nucleus. Other explanations are the same as those for FIG. 1.

TABLE 1. CELL BODY AND NUCLEAR SIZES, AND TOTAL NUMBER OF AVP-IMMUNOREACTIVE NEURONS

	Control (n = 5)		*Hypox (n = 4)*	
	Young	*Old*	*Young*	*Old*
Supraoptic nucleus				
Cell body size (μm^2)	107.7 ± 3.0[a]	115.5 ± 3.8	110.2 ± 3.7	99.2 ± 6.6
Nuclear size (μm^2)	45.4 ± 1.3	43.7 ± 1.1	38.1 ± 0.8**	39.2 ± 1.4
Total no. of neurons	4285 ± 232	2931 ± 341†	1201 ± 53**	1130 ± 204**
Paraventricular nucleus				
Cell body size (μm^2)	114.4 ± 3.4	103.0 ± 3.3	96.1 ± 1.5**	99.0 ± 4.2
Nuclear size (μm^2)	47.4 ± 1.0	44.1 ± 1.3	33.5 ± 1.7**	35.8 ± 0.8**
Total no. of neurons	1627 ± 90	1000 ± 85††	661 ± 100**	604 ± 146*

[a]Mean ± standard error.
Young: 3-month-old, Old: 19-month-old, Hypox: 10 days after hypophysectomy.
Differences from young: †$P < 0.05$, ††$P < 0.01$.
Differences from control: *$P < 0.05$, **$P < 0.01$.
Probabilities derived from Kruskal-Wallis H and Mann-Whitney U tests for cell body and nuclear sizes, and Student's *t* test for total number of neurons.

in the cell body and nuclear sizes between hypophysectomized young and old mice. Hypophysectomy significantly decreased the cell body size only in the PVN of young mice. However, cell nuclear size was decreased after hypophysectomy in the PVN neurons of both young and old mice and in the SON neurons of young mice. Total number of AVP neurons in the SON and PVN was decidedly less in intact old mice than intact young mice. After hypophysectomy, total number of AVP neurons of both nuclei were significantly decreased in both young and old mice, but there was no significant difference between young and old mice (Table 1).

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TABLE 2. CELL BODY AND NUCLEAR SIZES, AND TOTAL NUMBER OF OXT-IMMUNOREACTIVE NEURONS

	Control (n = 5)		*Hypox (n = 4)*	
	Young	*Old*	*Young*	*Old*
Supraoptic nucleus				
Cell body size (μm^2)	111.0 ± 3.9[a]	123.2 ± 2.7	107.0 ± 1.5	120.6 ± 10.4
Nuclear size (μm^2)	41.3 ± 1.7	41.9 ± 1.4	40.1 ± 0.9	42.3 ± 0.8
Total no. of neurons	964 ± 91	740 ± 49††	791 ± 149	648 ± 92
Paraventricular nucleus				
Cell body size (μm^2)	120.3 ± 1.0	111.5 ± 10.6	103.3 ± 4.5**	103.3 ± 3.6
Nuclear size (μm^2)	44.1 ± 0.8	44.3 ± 1.3	39.8 ± 1.6	39.4 ± 2.6
Total no. of neurons	2103 ± 77	1319 ± 103††	1371 ± 143**	1238 ± 198

[a]Mean ± standard error.
Young: 3-month-old, Old: 19-month-old, Hypox: 10 days after hypophysectomy.
Differences from young: ††$P < 0.01$.
Differences from control: **$P < 0.01$.
Probabilities derived from Kruskal-Wallis H and Mann-Whitney U tests for cell body and nuclear sizes, and Student's *t* test for total number of neurons.

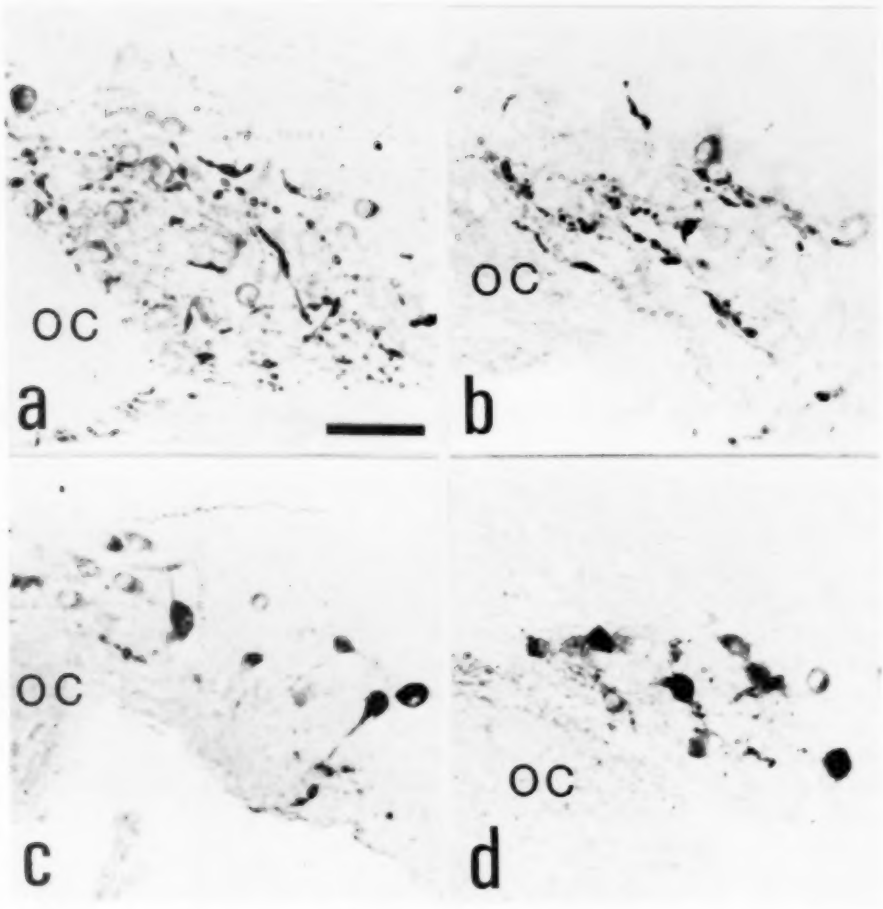

FIG. 3. Oxytocin-immunoreactive neurons in the supraoptic nucleus. Other explanations are the same as those for FIG. 1.

Oxytocin-immunoreactive neurons

In the SON, OXT-immunoreactive neurons (OXT neurons) were encountered dorsally in the anterior part. OXT neurons in the PVN were encountered generally in its anterior part and were present ventromedially in the posterior part. In intact old mice, the immunoreactivity of OXT neurons of the SON and PVN was somewhat weaker than in intact young mice, but the age-related decrease of stainability was less pronounced as compared with that in AVP neurons (Figures 3a, b and 4a, b). However, the reduction in the density of OXT fibers was observed in old mice likewise in AVP fibers.

After hypophysectomy, immunoreactivity was markedly intensified in a number of neuronal perikarya of the SON and PVN in both young and old mice (Figures 3c, d and 4c, d). The decrease of OXT fibers in the vicinity of neuronal perikarya of the SON and PVN was apparent in hypophysectomized young and old mice.

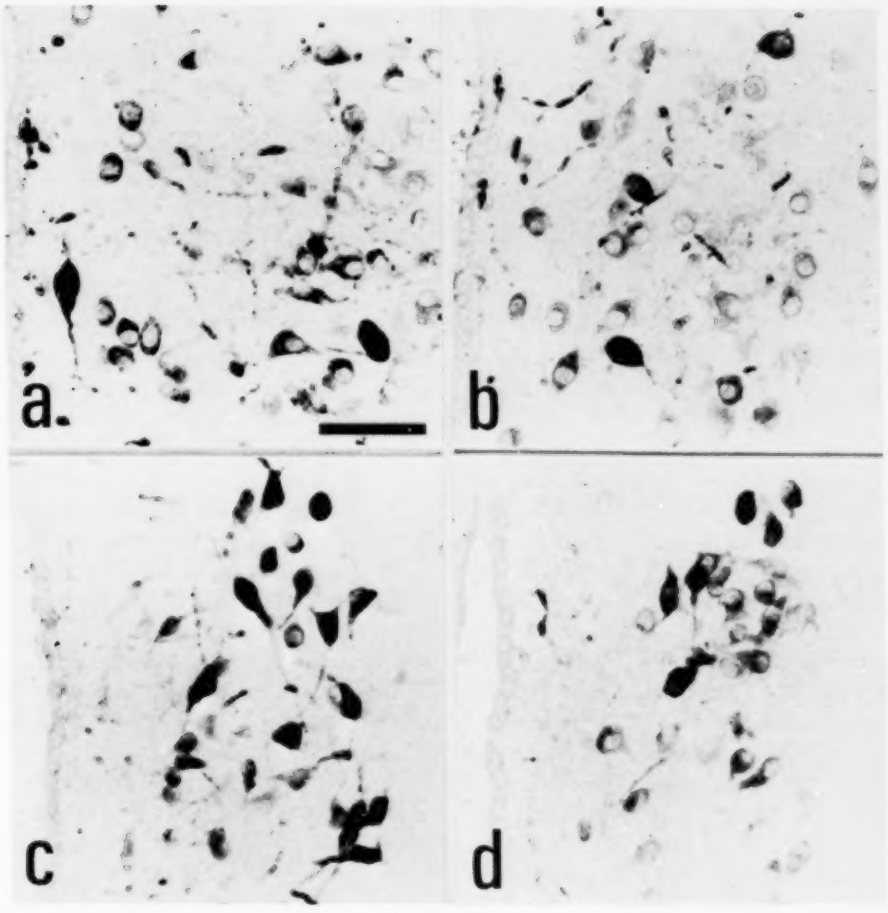

FIG. 4. Oxytocin-immunoreactive neurons in the paraventricular nucleus. Other explanations are the same as those for FIG. 1.

The results of morphometrical analyses of the cell body and nuclear sizes and total number of OXT neurons in the SON and PVN are shown in Table 2. There were no age differences in the cell body and nuclear sizes in both nuclei. Hypophysectomy tended to decrease the cell nuclear size, but the difference was not statistically significant unlike that in AVP neurons. Significant reduction after hypophysectomy was detected only in the cell body size of the PVN neurons in young mice.

In contrast, the total number of OXT neurons in the SON and PVN was significantly less in intact old than in young mice. Ten days after hypophysectomy the reduction in the total number of OXT neurons was detected in the PVN of young mice, but not in that of old animals. In addition, in the SON the total number of OXT neurons in hypophysectomized mice did not significantly deviate from that in the controls.

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DISCUSSION

In the present study, AVP neurons in the SON and PVN of old mice showed a marked reduction in immunohistochemical stainability to anti-AVP serum as compared with young mice, but the changes in OXT neurons were less conspicuous than in AVP neurons. These results were generally in agreement with those reported by Sladek *et al.* (1983) in rats at 20 and 30 months of age. In contrast, Watkins and Choy (1980) reported that stainability of OXT neurons was affected with age in the PVN more severely than in the SON. The total number of both AVP- and OXT-immunoreactive neurons was significantly less in intact old mice than those in intact young mice. However, analyses of Nissl-stained preparations revealed that the neuron number in the SON and PVN remained constant with age; the finding coincides with those of previous studies in rats (Hsu and Peng, 1978; Kawashima and Kobayashi, 1982; Peng and Hsu, 1982). These results indicate that the production of neurohypophyseal hormones, especially AVP, decreases with age. According to Sladek *et al.* (1983), in the AVP-deficient portion of the SON catecholaminergic innervation as visualized by histofluorescence was reduced in old rats. As norepinephrine exerts an inhibitory effect on the release of AVP (Armstrong *et al.*, 1982), Sladek *et al.* (1983) suggested that the reduced fluorescence innervation pattern shows a less active neuronal system at the synapses on the AVP neurons, but that the system may produce a basal level of AVP sufficient for the maintenance of water metabolism. Difference in neurosecretory activity between the SON and PVN in aged animals was also examined by Fliers and Swaab (1983). They showed that thiamine pyrophosphatase as an index of neurosecretory activity was similar in the SON between young and old rats but was increased in the PVN of old animals. By means of radioimmunoassay, age-related changes in the amount of AVP in the hypothalamus and the posterior lobe (PL) of the rat have been examined by several investigators. Sladek *et al.* (1981) reported that the amount of AVP in the PL was decreased in old rats of the Fischer 344 strain, but in the hypothalamus it was not significantly different from the level of young rats. According to Dorsa and Bottemiller (1982) in Long-Evans rats, and Zbuzek and Wu (1982) in Sprague-Dawley rats, significant decrease in the amount of AVP was found in the hypothalamus, but significant changes were not detected in the PL per unit weight basis. These inconsistent results may be due to the strain difference of the rat. In fact, two strains of rats maintained in our laboratory (Wistar/Tw and Sprague-Dawley/Tw) showed a great difference in the manifestation of age-related changes in neurosecretory activity (Kawashima and Kobayashi, 1982).

After hypophysectomy, axonal growth and eventual regeneration of a PL-like tissue take place at the cut end of the infundibular stalk. Five days after hypophysectomy AVP-containing fibers markedly increased in the external layer of the median eminence and at the stub of the stalk. Ten days after hypophysectomy, AVP fibers markedly increased, and the increase in stainability was more significant for anti-AVP serum than for anti-OXT serum (Kawamoto, 1985; Kawamoto and Kawashima, 1985). Similarly, the perikarya of AVP and OXT neurons in both SON and PVN were strongly stained 5 or 10 days after hypophysectomy. Although morphological changes further occur at more advanced postoperative days, initial state of polydipsia and polyuria observable at 5 days was significantly weakened at 10 days after the operation (Kawamoto, 1983). These results show that the neurosecretory system undergoing regeneration was functional at 10 days after hypophysectomy, and this time point was selected for the present study.

The immunostainability of AVP and OXT neurons was intensified in both SON and PVN in old mice 10 days after hypophysectomy, and the histological features were similar to those in hypophysectomized young mice. Hypophysectomy in rats and mice results in the decrease in the number of hypothalamic neurosecretory neurons and the significant variation in the amount of neurosecretory material among different neurons (Bodian and Maren, 1951; Moll and De Wied, 1962; Kawashima *et al.*, 1966; Kawamoto, 1985). The presence of intensely stained neurons may indicate that their axons were not completely sprouted to the release site in the reorganizing tissue and the synthesized neurohypophyseal hormones were stored in the perikarya. The present morphometric analyses also showed a decrease in the number of immunoreactive neurons in the SON decidedly and, to a lesser extent, in the PVN. The difference in the extent of decrease between the SON and PVN may relate to their difference in the axonal length from the cut end to the perikarya, on the assumption that the rate of retrograde degeneration was the same for neurons of both nuclei.

The number of AVP neurons appeared to be more strongly affected than that of OXT neurons in both young and old mice. These findings suggest that neurosecretory neurons, in particular AVP neurons of young mice, are composed of the population of neurons that are more readily affected by hypophysectomy than those of old mice. Thus, approximately the same number of immunoreactive neurons was observed in both young and old mice. These neurons are responsible for the reorganization of the PL after hypophysectomy. However, as we have reported in a separate paper (Kawamoto and Kawashima, 1985), the increase in the number of AVP fibers in the external layer of the median eminence 10 days after hypophysectomy was greater in 3-month-old mice than that observed in 19-month-old ones, suggesting a decrease in axonal plasticity in older mice.

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PUBLICATIONS OF INTEREST

B.B. BOGOMOLETS

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Cell Ageing and Cell Death

I. Davies and D.C. Sigee, Editors. Cambridge, MA: Cambridge University Press, 1984, 362 pages. ISBN 0-521-26172-4, $30.00.

This is the 25th Seminar Series of the Society for Experimental Biology and is devoted to the subject *Cell Ageing and Cell Death*. Held at the University of Birmingham in January 1984, it was jointly sponsored by the British Society for Research on Ageing. The contributed chapters are based on the organizers' belief that the primary mechanisms of aging lie at the cellular or molecular level. Not a bad idea. Only 1 of the 18 chapters is by a foreigner.

Review of Biological Research in Aging, Volume 2

M. Rothstein, Editor. New York: Alan R. Liss, Inc., 1985, 572 pages. ISBN 0-8451-3501-5, ISSN 0736-5055, $96.00.

The second in a series consisting of chapters authored by distinguished researchers. Coverage includes theories, evolution, genetics, immunology, neurobiology, endocrinology, cell biology, metabolism, drug metabolism, and mathematical modeling. This is one of at least three annual or periodical reviews of research in biogerontology. Is there really that much new and original work going on in this field to justify so many reviews? Is there a dull sameness about much that is being written?

Modifications of Proteins During Aging

R.C. Adelman and E. E. Dekker, Editors. New York: Alan R. Liss, Inc., 1985, 134 pages. ISBN 0-8451-2307-6, LC 85-12922, $32.00.

Proceedings of the mini-symposium session "Impact of Aging on Biochemical Function," held during the 75th Annual Meeting of the American Society of Biological Chemists, St.

Louis, Missouri, June 1984. Eight chapters with an introduction by the second Director of the National Institute on Aging, T. Franklin Williams. Many enzymes and other proteins become modified during the aging process. Those that are covered here include deamination, phosphorylation, glycosylation, carboxylation, mixed function oxidation reactions, and noncovalent transition.

Cellular and Molecular Aspects of Aging: The Red Cell as a Model

J.W. Eaton, D.K. Konzen, and J.G. White, Editors. New York: Alan R. Liss, Inc., 1985, 476 pages. ISBN 0-8451-5045-6, LC 85-13197, $68.00.

Proceedings of a conference held in Minneapolis, Minnesota, September 1984. The erythrocyte, they say, is an invaluable model for aging because of its accessibility (not a good reason), its limited in vivo life span (a better reason), and the numerous documented changes that occur over time (the best reason). Thirty chapters, many authored by leaders in the field. Bloody good book.

Molecular Basis of Aging

A.K. Roy and B. Chatterjee, Editors. New York: Academic Press, 1984, 400 pages. ISBN 0-12-601060-9, $29.50.

These articles are based on a symposium held at Oakland University, Rochester, Michigan in November 1983. A multi-authored work by some of the stars in the field. Somewhat repetitious of previously published work but useful for the frequent lively discussions that are reproduced after most chapters.

Tissue Culture of Epithelial Cells

M. Taub, Editor. New York: Plenum Press, 1984, 288 pages. ISBN 306-41740-5.

For those who thought that only fibroblasts grow well in vitro this book should be an eye-opener. Aging at the cell level is not restricted to fibroblasts. Now that techniques are available for the easy cultivation of epithelial cells, new insights into cell aging will surely be forthcoming. Like fibroblasts, cultured normal epithelial cells also lose functional ability and ultimately age and die. It has been recently discovered that about 100 years ago August Weismann was first to speculate that aging may result from the loss of proliferative and functional capacity in normal cells. Is there nothing new?

Handbook of Cell Biology of Aging

V.J. Cristofalo, Editor. Boca Raton, FL: CRC Press, Inc., 1985, 640 pages. ISBN 0-8493-3142-0, $138.00.

Cell aging in most parts of the anatomy, including the nervous system, skeletal muscle, liver, kidney, intestine, reproductive system, mammary tissue, endothelium, erythrocytes, immune system, lung and skin is reviewed. No cardiology—a heartless omission. Cells in their generality are discussed. This includes changes in number, cycle, and morphology. Nematodes, *drosophila*, and other insects bring up the rear. Unlike another book with an almost identical title and published in the same year (see next item) this one has 400 fewer pages. But is it a handbook? According to *Webster's Ninth New Collegiate Dictionary*, a handbook is "A book, especially a reference book that can be easily handled or consulted; a manual." Enough said. The 640 pages will cost you about 21 cents per page, whereas its 1025-page competitor costs 7 cents a page. There must be a message here.

Handbook of the Biology of Aging

C.E. Finch and E.L. Schneider, Editors. New York: Van Nostrand Reinhold, 1985, 1025 pages. ISBN 0-442-22529-6, $75.00.

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A second edition of the popular trilogy first published 7 years ago. It is the only one of the three that has had one of its original editors replaced. No explanation given. Covers the same ground as its competitor described in the previous review, but includes epidemiology, evolution, plant senescence, cytogenetics, longitudinal studies, pharmacology, arteriosclerosis, hypertension, nutrition, sleep and health maintenance. "Geriatric Medicine" is covered in 27 pages. Unlike its competitor, this one has a heart—thanks to E.G. Lakatta. At least 2 of the 58 people listed as "contributors" have not authored papers. Weighing in at a bit under 2 kilos, this is no handbook but at 7 cents a page it may be a bargain.

Developments in Aging: 1984, Volumes 1 and 2

U.S. Senate Committee on Aging, 99th Congress, 1st Session. Report 99-5, Vols. 1 and 2. Washington, DC: U.S. Government Printing Office, 1985, 348 pages, Vol. 1; 408 pages, Vol. 2.

These volumes represent the annual report of the U.S. Senate's Special Committee on Aging. They spent $1,159,720 on their 1984 activities as summarized in these 750 pages. The first volume embraces retirement income, low-income assistance programs, health, housing, and social services. Volume 2 contains reports from dozens of federal departments and agencies, among which are such notable gerontology units as the U.S. Forest Service,

the Department of Defense, the Office of the Inspector General, and the Civil Aeronautics Board. The latter simply say that they have promulgated a new rule prohibiting an airline from denying passage to a ticket holder because of age. The Patent and Trademark Office offers "accelerated" examination of any patent application from a citizen 65 or older. Because both patents and 65-year-olds have a life expectation of 17 years it really does make perfect sense.

Technology and Aging in America

Office of Technology Assessment, U.S. Congress, Washington, DC, OTA-BA-264. Washington, DC: U.S. Government Printing Office, 1985, 496 pages. Stock No. 052-003-00970-6, $17.00.

High tech finally comes to biogerontology, courtesy of the Congressional Office of Technology Assessment. According to their distinguished advisory panel, new technologies can improve the functional abilities of older persons and possibly reduce health care expenditures—a bold and unexpected conclusion. Five chronic conditions are assessed: osteoarthritis, dementia, osteoporosis, hearing impairments, and urinary incontinence. Detailed appendices are provided on health status, nutrition, cell biology of aging, and applications of biotechnology to diagnosis and treatment of disease.

Prolongevity II

A. Rosenfeld. New York: A.A. Knopf, 1985, 336 pages. ISBN 0-394-534755-1, $18.95.

An updated version of this popular book intended for a general audience. Clearly, it is the most authoritative and well written summary of the current status of research in biogerontology. Strongly recommended as the only credible overview of the field written for the layman. In fact, beginning students in biogerontology and also practicing biogerontologists will benefit from what certainly must be one of the best "reads" in the field.

Cellular Ageing

H.W. Sauer, Editor. Basel, Switzerland: S. Karger, 1984, 276 pages. ISBN 3-8055-3860-X, $88.75.

Chapters are authored by 18 candidates for the 20th annual Heinz Karger Memorial Foundation competition. The subject was cellular aging. An edited book, but the usual motivation for submitting papers did not come from a strong-willed editor. The opportunity to win 20,000 Swiss franks inspired the submissions. A commendable book some-

what diminished by the perpetuation of several inaccuracies in the beginning overview authored by the only non-candidate. At $88.75 the book sells for about one-half of its weight in pure silver.

Normal Human Aging: The Baltimore Longitudinal Study of Aging

N.W. Shock. U.S. Department of Health and Human Services, NIH Publication No. 84-2450. Washington, DC: U.S. Government Printing Office, 1984, 434 pages, $18.00.

The definitive work on the history and present activities of the highly respected Baltimore Longitudinal Study by the distinguished gerontologist who, among other triumphs, put this all together. Two-thirds of the book consists of an appendix in which the full text of previously published articles on the study have been reproduced. One of the most valuable books in gerontology to appear in recent years.

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ANNOUNCEMENT

The Third Tokyo Symposium on Liver and Aging

THE THIRD Tokyo Symposium, *Liver and Aging*, will be held in Tokyo, Japan on August 20 through 22, 1986 with additional coverage of *Liver and Brain*. The primary objective of the symposium is to discuss the interactions and functional alterations of these two organs as they are affected by aging. The symposium will be limited to about 100 participants. The total number of speakers will be less than 30.

Though participation will generally be limited to predetermined invitees, a small number of interested and qualified guests will be admitted both as audience and speakers. Financial support will be provided for all invited speakers from outside Japan. Those who wish to present papers are encouraged to submit an abstract to the organizer. Papers most appropriate to the subject will be given priority.

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The majority of the presentations will be on the pharmacodynamics of drugs affecting the central nervous system, including drug metabolism, pharmacokinetics, end organ sensitivity changes during aging, and neural and humoral control of the aging liver. Papers covering the aging of only a single organ (liver or brain) may be included but the number of such papers will be limited.

Those interested in the symposium are kindly requested to contact the organizer.

THIRD TOKYO SYMPOSIUM *LIVER AND AGING* and *LIVER AND BRAIN*

Place: Hotel Pacific, Takanawa, Tokyo, JAPAN

Date: August 20–22, 1986

Abstract Deadline: February 28, 1986

Organizer: K. Kitani, M.D.
First Laboratory of Clinical Physiology
Tokyo Metropolitan Institute of Gerontology
35-2, Sakaecho, Itabashi-ku
Tokyo, JAPAN (Telephone 03-964-1131, Ext. 3091)

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One of today's growing concerns...

Geriatric Drug Use

Clinical and Social Perspectives

Published in cooperation with the Drug Information Association

Editors:

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Recent medical advances have increased our lifespans. We have an expanding population of the aged. One of our greatest concerns is the role medication plays in serving the needs of this fast growing segment of today's society. **GERIATRIC DRUG USE — CLINICAL AND SOCIAL PERSPECTIVES** summarizes the proceedings of the Drug Information Association workshop held February 27-28, 1984 in Washington, DC. The text highlights significant new findings regarding drug utilization studies in the treatment of the elderly.

Partial Contents:

- ★ Clinical and Social Perspectives on Geriatric Drug Use, **C. E. Koop & L. Tolliver**
- ★ Issues in Geriatric Labeling Revisions, **L. Millstein**
- ★ Self-Medication with Over-the-Counter Drugs, **A. Eckian**
- ★ The World Health Organization's Global Program of Health Care for the Elderly, **M. Dukes**
- ★ Adverse Drug Reaction Surveillance, **S. Moore and J. Jones**
- ★ Survey of Drug Use and Mental Disorders in Nursing Homes, **M. Harper**
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- ★ Pharmacokinetic Risk Factors in the Elderly, **D. Greenblatt, D. Abernathy, R. Shader**
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THE EFFECT OF ENDURANCE EXERCISE ON BONE DIMENSIONS, COLLAGEN, AND CALCIUM IN THE AGED MALE RAT

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Abstract—Sixteen weeks of a relatively mild running program, started at 22 months of age, lowered the body weights of 26-month-old male rats to the level of 9-month-old rats and increased the weights and the collagen densities of hind limb bones to levels greater than those of 9-, 22-, and 26-month-old sedentary rats. The densities (g/cm³) and the calcium densities (mg/cm³) of the hind limb bones decreased with age and were restored to the 9-month level by training the elderly rats to run. These data suggest that exercise is capable of inducing a compensation for, or a reversal of, age-associated bone loss (osteoporosis) and restoring the bone mineral content in aged rats to the level of those of mature young adult animals.

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INTRODUCTION

ONE OF the most consistent physiological changes that accompanies aging in vertebrates is a progressive decrease in bone mineral content, strength, and resistance to fracture. Such changes are common in most human populations and types of bone (Garn, 1970; Avioli, 1981) and the susceptibility to bone fracture appears to be directly proportional to the degree of bone loss (McMillan, 1978). Bone mineral decreases at a rate of between 0.75 and 1% per year beginning at age 35–45 for women and 0.4–0.5% per year starting at age 50–55 for men, resulting in a 50-fold increase in hip fracture between 40 and 70 years of age (Chalmers, 1970). In humans with senile osteoporosis, fractures are most commonly associated with the radius, ulna, femoral neck and vertebral column (Arnold *et al.*, 1969) and may occur spontaneously or as the result of mild trauma (Alffram and Bauer, 1962). Complication from fractures in postmenopausal women has resulted in a mortality rate of 15 to 30%, with an estimated annual cost greater than 1 billion dollars (Avioli, 1981). The emotional, medical, and economic costs related to bone deterioration are enormous and will surely become greater as the number of elderly continues to increase.

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Although there appears to be general agreement that some bone loss invariably accompanies aging (Albanese *et al.*, 1969; Cassuccio, 1962; Garn *et al.*, 1966, 1967, 1969; Goldsmith *et al.*, 1971; Rose, 1970; Smith, 1967; Smith *et al.*, 1981b; Urist, 1962), the etiology and mechanism are still speculative and methods employed for prevention and treatment have met with mixed success (Smith *et al.*, 1981b; Avioli, 1977; Montoye *et al.*, 1976). Of all the treatment modalities employed for osteoporosis, physical activity has received the least support, despite reports that in athletes and physical laborers bone hypertrophy related to their specific activities occurs (Smith, 1981; Montoye *et al.*, 1980; Krølner and Toft, 1983; Smith *et al.*, 1981b) and that exercise may stimulate bone accretion in humans (Mazess and Whedon, 1983; Krølner *et al.*, 1983; Brewer *et al.*, 1983; Oyster *et al.*, 1984; Williams *et al.*, 1984) and rodents (Bell *et al.*, 1980; Saville and Whyte, 1969).

Most studies on senile osteoporosis have focused on females because of their earlier development of symptomatology. However, an increase in the incidence of osteoporosis in males may be expected as the extension in life expectancy results in an elevation in the population of elderly men. In order to gather additional information on the ability of endurance exercise to prevent or reverse osteopenia in the aged male animal, we have analyzed the effect of a relatively mild regimen of endurance training on the physical dimensions, as well as calcium and collagen contents, of hindlimb bones of elderly male rats and have compared such values to those of age-matched and middle-aged sedentary control groups.

MATERIALS AND METHODS

Animals

Retired male breeder Sprague-Dawley rats****, designated Crl:COBS[R]CD[R] (SD), were obtained from Charles River Breeding Laboratories (Portage, MI) at 9 to 10 months of age and housed singly in the Animal Care Facility of the Division of Biological Sciences, University of Michigan, in a room segregated from other species and maintained at 22°C on a 12 h light-dark cycle. All animals received Purina Laboratory Rat Chow containing 1.01% calcium and 0.74% phosphorus and water ad libitum. Approximately 85% of the rats in our aging colony survive to 26 months of age. Four groups of rats, selected at random from our aging colony, were prepared: (a) 9-month-old sedentary, (b) 22-month-old sedentary, (c) 26-month-old sedentary, and (d) 26-month-old endurance-trained for 4 months. Sedentary animals were handled by laboratory personnel for several minutes each, 5 days per week, in order to approximate the degree of handling of the endurance-trained group. Because it was our intent to study the interaction of age and exercise, animals that showed pathology or unexplained weight loss were excluded from participation in these experiments.

Animal training

The exercise training protocol was similar to that described previously (Starnes *et al.*, 1983a,b). A group of 22-month-old rats was introduced into a protocol consisting of running 5 days per week for 16 weeks in a 10-channel, motor-driven treadmill (Quinton Instruments, Seattle, WA). Throughout the exercise program, the angle of incline of the treadmill was 8 ° and the temperature in the exercise room was maintained at 19° C. The first exercise session consisted of running the rats for 10 minutes at 11 m/min. On each succeeding session for the next 2 weeks, the duration and speed were increased until the rats were running for 40 minutes at 22 m/min. For the remainder of the training period, the rats ran an average of 35 minutes each day.

****Personal contact between the first author and the manager of the local rodent breeding facility enabled us to purchase retired breeders of known birth dates for these studies.

Chemicals

All chemicals used were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO) except for *p*-(dimethylamino)-benzaldehyde obtained from J.T. Baker Co. (Phillipsburg, NJ) and murexide (acid ammonium purpurate) from Fisher Scientific Co. (Pittsburgh, PA).

Tissue preparation

All animals were anesthesized with sodium pentobarbital and gastrocnemius, quadriceps, and cardiac muscles were removed and used for other purposes (Beyer *et al.*, 1984). Carcasses were stored frozen in air-tight containers until investigators were available for bone analyses (between 3 and 4 months), when right and left femurs and tibias were removed. After mechanical removal of most of the soft tissue, the bones were cleansed thoroughly of adhering tissue by the domestic beetle, *Dermestes vulpinus*. Bones were then washed with distilled water for 5 minutes, immersed in 10% NaCl for 48 hours, and rinsed again in distilled water to remove any traces of adhering connective and adipose tissue.

Physical measurements

Bone volume was determined according to Archimedes' Principle ($F = pgV$) by degassing the bone in distilled water *in vacuo* for 60 minutes, obtaining the buoyancy force from the difference in the weight of a beaker of distilled water, with and without the bone immersed, while suspended by a fine copper wire and calculating the volume of the bone essentially as described by Dequeker and Merlevede (1971). Density was calculated as dry weight/volume. This parameter should not be confused with the numerical value obtained with X-ray densitometry; the latter technique derives values more closely related to porosity of bone. Lipid was extracted from bones by the Soxhlet apparatus with petroleum ether (30–60 ° BP) for 60 hours. Fat-free dry weight was obtained after dehydrating the bone *in vacuo* at 108 ° C for 60 minutes.

Chemical analyses

Bones were prepared for calcium analysis according to Kenny *et al.* (1958). Calcium was analyzed in femurs either with a Perkin-Elmer Model 272 Atomic Absorption Spectrophotometer (Perkin-Elmer Corp., Norwalk, CT), using the method described in the manufacturer's handbook, or colorimetrically using the EDTA-murexide method of Munson *et al.* (1955) as refined by Ohnishi and Ebashi (1964). The two methods yielded comparable results. Collagen content of finely crushed tibias were determined by analyzing for hydroxyproline essentially according to Miyada and Tappel (1956), with the acid hydrolysis procedure of Neuman and Logan (1950), the modifications suggested by LeRoy (1967), and the corrections suggested by Dequeker and Merlevede (1971). The terms *calcium density* and *collagen density* are defined as mg calcium/cm^3 and mg collagen/cm^3 bone, respectively.

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Statistical analysis

All data obtained were analyzed by a univariate one-way analysis of variance (ANOVA) using the Michigan Interactive Data Analysis System (MIDAS) developed by the Statistical Research Laboratory of The University of Michigan. Inferences about the significance of the differences between means, as well as conservative Scheffe allowances, were derived from such values.

RESULTS

The group of rats participating in the exercise program lost 10% of their body weight during the first 7 weeks of the program ($P < 0.001$), after which their weights remained relatively stable (Beyer *et al.*, 1984). At the end of the 16 weeks of exercise the rats in the elderly trained group weighed 16.1% less than their age-matched sedentary controls ($P < 0.0001$). The mean body weight of the 26-month-old trained group did not differ from that of the 9-month-old control group ($P = 0.7125$). The continual weight gain of this strain of Sprague-Dawley rat over its life span has been documented previously (Farrar *et al.* 1981; McCafferty and Edington, 1974).

Training also resulted in an increase in the dry and fat-free dry weights of femurs and tibias to levels equivalent to or greater than the 9-, 22- and 26-month-old sedentary rats

TABLE 1. PHYSICAL DIMENSIONS AND CALCIUM ANALYSES OF FEMURS FROM 9-, 22-, AND 26-MONTH-OLD SEDENTARY AND 26-MONTH-OLD ENDURANCE-TRAINED MALE RATS

	Experimental group					*Statistical P values*				
Femur Parameter	Sed-9	Sed-22	Sed-26	Tr-26	ANOVA	Sed-9 vs Sed-26	Sed-9 vs Tr-26	Sed-22 vs Tr-26	Sed-26 vs Tr-26	Sed-22 vs Sed-26
Dimensions, length, mm	40.33 ±.36(12)	42.79 ±.49(12)	42.93 ±.73(12)	42.63 ±.47(12)	.0031	.0012[a]	.0038[a]	.8350	.6934	.8522
midshaft diam., mm	5.09 ±.09(12)	4.98 ±.10(12)	4.96 ±.11(12)	5.18 ±.12(12)	.4192	.3709	.5750	.1820	.1490	.9105
Dry wt., g	.9501 ±.03(12)	.9254 ±.03(12)	.9205 ±.03(12)	1.1060 ±.03(12)	.0001	.4904	.0007[a]	.0001[a]	.0001[a]	.9084
Fat-free dry wt., g	.9052 ±.029(12)	.8997 ±.033(12)	.8828 ±.029(12)	1.0317 ±.032(12)	.0053	.6134	.0063[a]	.0045[a]	.0015[a]	.7040
Volume, cm^3	.8783 ±.025(12)	.9476 ±.024(12)	.9868 ±.025(12)	.9995 ±.031(12)	.0098	.0056[a]	.0022[a]	.1702	.7337	.2986
Density, g/cm^3	1.0822 ±.021(12)	.9583 ±030(12)	.9330 ±.023(12)	1.1095 ±.014(12)	<.0001	<.0001[a]	.4004	<.0001[a]	<.0001[a]	.4335
Total Ca^{++}, mg	274.1 ±7.5(11)	257.7 ±5.8(12)	258.1 ±7.5(10)	303.0 ±6.5(11)	.0001	.1130	.0046[a]	<.0001[a]	<.0001[a]	.9645
Ca^{++}/dry wt., mg/g	282.7 ±7.8(12)	283.8 ±5.29(12)	282.9 ±7.9(10)	272.7 ±5.3(11)	.6095	.9805	.2888	.2402	.2995	.9290
Ca^{++} density, mg Ca^{++}/cm^3	305.2 ±7.8(12)	266.8 ±5.8(12)	260.7 ±5.2(10)	301.4 ±6.0(11)	<.0001	<.0001[a]	.6729	.0004[a]	.0001[a]	.5075

[a]This difference is also significant at the .05 level of confidence using Scheffe-type simultaneous coefficient intervals.

Values are mean ± 1 standard error of the mean. Parentheses contain the number of animals in each group. Statistical *P* refers to the probability that the difference between the means of two groups is due to chance alone.

TABLE 2. PHYSICAL DIMENSIONS AND COLLAGEN ANALYSES OF TIBIAS FROM 9-, 22-, AND 26-MONTH-OLD SEDENTARY AND 26-MONTH-OLD ENDURANCE-TRAINED MALE RATS

	Experimental group					*Statistical P values*				
Tibia Parameter	Sed-9	Sed-22	Sed-26	Tr-26	ANOVA	Sed-9 vs Sed-26	Sed-9 vs Tr-26	Sed-22 vs Tr-26	Sed-26 vs Tr-26	Sed-22 vs Sed-26
Dimensions, length, mm	46.37 ± .18(12)	47.31 ± .48(12)	47.76 ± .47(12)	48.63 ± .22(12)	.0009	.0103[a]	.0001[a]	.0143	.0990	.3908
midshaft diam., mm	4.01 ± .06(12)	4.29 ± .14(12)	4.39 ± .06(12)	4.50 ± .04(12)	.0101	.0112[a]	.0015[a]	.1570	.4580	.4931
Dry wt., g	.6315 ± .01(12)	.7027 ± .02(12)	.7133 ± .02(12)	.8184 ± .01(12)	<.0001	.0010[a]	.0001[a]	.0001[a]	.0001[a]	.6478
Fat-free dry wt., g	.6018 ± .01(12)	.6784 ± .02(12)	.6877 ± .02(12)	.0771 ± .01(12)	<.0001	.0001[a]	.0001[a]	.0001[a]	.0001[a]	.2986
Volume, cm^3	.5754 ± .01(12)	.6909 ± .02(12)	.7194 ± .02(12)	.7148 ± .01(12)	<.0001	.0001[a]	.0001[a]	.2466	.8192	.2466
Density, g/cm^3	1.1037 ± .02(12)	.9870 ± .02(12)	.9929 ± .02(12)	1.1454 ± .01(12)	<.0001	.0001[a]	.1148	<.0001[a]	<.0001[a]	.8203
Total collagen, mg	103.9 ± 1.7(11)	126.9 ± 3.9(12)	129.6 ± 2.9(11)	154.6 ± 1.9(11)	<.0001	<.0001[a]	.0001[a]	<.0001[a]	<.0001[a]	.5156
Collagen/fat-free dry wt., mg/g	172.4 ± 2.6(12)	187.0 ± 4.4(12)	191.1 ± 3.5(11)	197.6 ± 2.2(11)	<.0001	.0003[a]	.0001[a]	.0283	.1784	.3872
Collagen density, mg/cm^3	181.2 ± 1.9(12)	178.2 ± 3.6(12)	182.3 ± 3.8(11)	215.0 ± 2.8(11)	<.0001	.8013	<.0001[a]	<.0001[a]	<.0001[a]	.3525

[a]This difference is also significant at the .05 level of confidence using Scheffe-type simultaneous coefficient intervals.

Values are mean ± 1 standard error of the mean. Parentheses contain the number of animals in each group. Statistical *P* refers to the probability that the difference between the means of two groups is due to chance alone.

(Tables 1 and 2). Indications of the occurrence of osteopenia in these aging animals are the significant decreases in femur and tibia densities (g/cm^3) (Tables 1 and 2) and the decrease in femur calcium density (Table 1), a reflection of the amount of calcium per volume of bone. This loss in bone mineral density occurred despite increases in bone volume, total collagen, and collagen content per fat-free dry weight and without an associated decrease in collagen density between 9 and 26 months (Table 2).

The data in Tables 1 and 2 thus indicate that the effects of exercise on the hind limb bones in these elderly rats were (a) an increase in dry weights and fat-free dry weights, (b) an increase in the total bone density to the level seen in the 9-month-old sedentary rat, (c) an increase in total calcium to levels greater than the 9-, 22-, and 26-month sedentary age groups, (d) a compensation for, or a reversal of, the loss of calcium density, due to senile osteoporosis, to a level not statistically different from 9-month-old sedentary animals, and (e) significant increases in total collagen and collagen densities.

DISCUSSION

The data from this study suggest that the decrease in hind limb bone weight with age is due primarily to loss of mineral content and not to the loss of organic bone material. The loss of total calcium and dry weight between 9 and 22 to 26 months are quantitatively similar when an adjustment is made for the molecular form of calcium in bone—hydroxyapatite. As the percentage increase in total collagen and bone volume were parallel, no change in collagen density was observed. Because of this increase in total collagen and diminished mineral content, the collagen per fat-free weight increased with age. This is in agreement with the report of Dequeker and Merlevede (1971) that collagen content of porous bone related to either fat-free weight or dry weight tends to increase with age. Thus, according to the present study, osteoporotic bone from aged animals has a lowered density when compared to 9-month-old animals, due primarily to loss of the mineral component, and an increased collagen fraction due to the greater loss of hydroxyapatite than to the collagen matrix.

Senile osteopenia appears to be a consequence of both an increased calcium demand due to negative calcium balance and loss of the calcium homeostasis of bone due to a break-down of regulatory mechanisms (Avioli, 1981). Failure to prevent the negative calcium balance inevitably results in substantial bone resorption, leading to low mass and high collagen concentrations. The present experiments demonstrate that net mineral loss does not occur in hind limb bones of physically active animals; to the contrary, total mass increases due to increases in both total collagen and minerals. Exercise thus appears to influence the formation of the connective tissue matrix essential for mineral deposition in bone formation. Exercise also stimulates the turnover of connective tissue in a variety of other tissues (Beyer, 1983).

Although the precise mechanism of bone loss in senile osteoporosis in not known, a number of processes have been implicated, including decreased intestinal and renal calcium absorption leading to a negative calcium balance, complex hormonal interactions, inactivity (Avioli, 1982), and the loss of calcium homeostasis due to degeneration of regulatory systems on the bone surface (Tonna, 1978; Tonna and Cronkite, 1964). Because endurance exercise appears to compensate for the loss of bone in the aged animals, an appropriate approach to the study of underlying metabolic bases for the prevention of osteoporosis may be to investigate such systems in animals adapting to endurance exercise. It has been reported that osteoblastic activity and population decrease dras-

tically with advancing age (Tonna, 1978; Tonna and Cronkite, 1964). Observations of osteoblasts during endurance training in the elderly animal, therefore, may yield valuable information on control of osteoblastic activity, as well as its possible role in bone repair in the elderly.

A bone morphogenetic protein (BMP) that may regulate the differentiation of mesenchyme cells into cartilage and bone has been demonstrated (Urist *et al.*, 1979) and found to be diminished in aged bone (Urist *et al.*, 1981). This protein may be involved in the response of bone to exercise reported herein. Because the present study indicates that exercise increases bone density by stimulating matrix formation in partially demineralized bone and because BMP is assayed by its ability to induce osteogenesis by demineralized bone matrix (Syftestad and Urist, 1980), a study of the activity of BMP in the bones of exercising individuals may yield important information. These and other factors that affect bone have been reviewed recently (Simpson, 1984).

As Hayflick (1979) has suggested, a decline in proliferative potential is an innate, programmed property of all cells. One of the goals of those studying aging is to identify means of shifting the aging curve from a general decline to a more rectilinear shape. Many of the changes that accompany aging, at least in the laboratory rat and the human, are similar to those accompanying disuse atrophy (Bortz, 1982). The data reported herein and elsewhere suggest that a regular exercise program is able to reverse, or delay, some of the age-related functional declines, even if initiated late in the life of otherwise sedentary individuals. This is not to suggest, however, that cells remain responsive indefinitely to stimuli such as exercise because some evidence exists for a "threshold age" (Edington *et al.*, 1972). Richardson and Cheung (1982) have suggested that with age there occurs a general decline in gene expression, translation, and transcription, resulting in a decrease in the response of the aged animal to stimuli and a lowering of induced enzyme synthesis. Although it appears that tissues of animals close to the end of their maximal life span are unresponsive to stimuli such as exercise (Steinhagen-Thiessen *et al.*, 1980, 1981), those of somewhat younger animals are still able to reverse degenerative age-associated metabolic and structural changes. However, the question of the fraction of the life span of an animal that must remain in order for aging-associated degenerative changes to maintain responsiveness to repair stimuli is still unanswered. The data reported herein suggest that relatively mild endurance exercise, inititated in animals in which osteoporosis has progressed to a measurable level, may still reverse or compensate for this disabling, degenerative condition of old age. In a study of women averaging 81 years, exercise over a period of 3 years has been shown to slow the rate of bone loss and increase bone mineral content (Smith *et al.*, 1981b). Apparently, 81 years in the human and 22 months in the rat are not too late to start employing exercise to protect bones from the adverse effects of aging and the consequences of a sedentary lifestyle.

Bone loss is a well-recognized consequence of bed rest, limb immobilization, and loss of normal gravitational stimuli due to space travel (Smith, 1981; Montoye *et al.*, 1980; Krølner and Toft, 1983; Smith *et al.*, 1981a; Wronski and Morey, 1983). Data also exist demonstrating prevention of space travel-associated bone loss with appropriate exercise (Mack *et al.*, 1967) as well as reversal of bone loss in elderly animals by exercise (Saville and Whyte, 1969; Nilsson and Westlin, 1971; Smith *et al.*, 1976; Bell *et al.*, 1980; Lanyon and Bourn, 1979; Krølner *et al.*, 1983). For these reasons this non-invasive treatment, exercise, lacking the side effects of alternative drug therapy, should receive serious, widespread consideration. A plea for more information in this area through research on humans has been published (Holloszy, 1983).

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It should be of interest to note that other measurements on the rats used in this study or others similarly trained indicate that gastrocnemius muscle myoglobin (Beyer and Fattore, 1984), flavin, and oxidative enzymatic capacities (Beyer *et al.*, 1984) are all increased in the trained aged group to levels equal to, or greater than, those of the 9-month-old sedentary animals. In addition, myocardial function and aerobic energy metabolism were improved (Starnes *et al.*, 1983a) and coenzyme Q concentrations increased (Beyer and Starnes, 1985) when compared to age-matched sedentary animals.

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ACCUMULATION OF HEAT-LABILE ELONGATION FACTOR 2 IN THE LIVER OF MICE AND RATS

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Abstract—Age-related changes of polypeptide chain elongation factor 2 (EF-2) in mouse and rat livers were investigated. The percentage of heat-labile components in EF-2 was 5 to 10% in young animals and 15 to 45% in old ones. These results and other findings (Takahashi *et al.*, in press) support the idea that decrease in translational activity in old animals is due, in part, to alterations of protein components of the translational system.

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INTRODUCTION

AGING OF animals is characterized by a gradual decrease in function of most cells and tissues. The decrease may be explained by alterations in synthesis and degradation of proteins, and in fact there are many reports that protein synthesis decreases with age (Blazejowski and Webster, 1983; Makrides, 1983; Mori *et al.*, 1979; Nakazawa *et al.*, 1984; Richardson and Cheung, 1982). It is suggested that altered proteins accumulate during aging mainly due to decrease in degradation (Gershon, 1979; Rothstein, 1979; Lavie *et al.*, 1982).

We previously reported that liver ribosomes from old mice showed 30–40% lower activity than those from young mice (Mori *et al.*, 1979) and that the activity for formation of an initiation complex of the 40 S ribosomal subunit was 15–20% lower in old mice than in young ones (Nakazawa *et al.*, 1984). These findings suggest that not only the initiation but also the elongation step of protein synthesis is affected by age. Two factors are required for elongation of polypeptide chains, elongation factor 1 (EF-1) for the binding of aminoacyl tRNA to ribosomes, and elongation factor 2 (EF-2) for the translocation of peptidyl tRNA.

Bolla and Brot (1975) showed, by an immunological method, that inactive or partially active EF-1 molecules accumulate in old nematodes. Moreover, Moldave *et al.* (1979) reported that EF-1 activities in the liver and brain are 30–40% higher in young rats than in old ones, but that the activity of EF-2 is similar in young and old animals. In *Drosophila melanogaster*, also, decrease in protein synthesis with age appears to be due mainly to lowered EF-1 activity (Webster and Webster, 1984).

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We examined alterations in the components of the translational apparatus during aging and recently found increases in heat-labile aminoacyl tRNA sythetases in the tissues of old rats (Takahashi *et al.*, in press). In this work, we found that there is more heat-labile EF-2 in the liver in old mice and rats than in young ones.

MATERIALS AND METHODS

Animals

Inbred male ddY mice and male Wistar STD rats were used. The animals were obtained from Sankyo Laboratory, Tokyo, and maintained at 22 °C in an atmosphere of 55% relative humidity under a constant daily 12 h light–12 h dark cycle. They were given a commercial pellet diet (CE-7, Nippon Clea Co., Tokyo) and water *ad libitum*. Under these conditions, the mean and maximum life spans of mice were 18 months and 29 months, respectively, as reported previously (Mori *et al.*, 1978), and the mean life span of rats was 20 months.

Chemicals

ATP was purchased from Boehringer-Mannheim. Phosphocreatine, creatine phosphokinase, and polyuridylate [poly(U)] were from Sigma. Sephadex G-200 was from Pharmacia Fine Chemicals. DEAE-cellulose (DE 52) was from Whatman and hydroxylapatite was from Nippon Chemicals. L-[^{3}H] phenylalanine (specific activity 21 Ci/mmol) was from the Radiochemical Centre, Amersham, England. All other reagents were of reagent grade.

Preparation of ribosomes from rabbit reticulocytes

The ribosomes used for assay of elongation factors were prepared from rabbit reticulocytes by the method of Falvey and Staehelin (1970), as modified by Mori *et al.* (1979). Briefly, male or female rabbits were made anemic by subcutaneous injection of β-acetylphenylhydrazine. Polysomes were obtained from their reticulocyte lysate by centrifugation and treated with puromycin and KCl to remove mRNA, tRNA, and translational factors. The purified ribosomes were stored at −80 °C until use. The post-polysomal supernatant (S-100) was saved to prepare elongation factors (see below).

Preparation of elongation factors from mouse and rat livers

EF-2 of mouse liver was prepared as described by Kemper and Merrick (1979) with slight modifications. For comparison of the heat-stabilities of EF-2 from young and old animals, the preparations were purified in parallel to reduce variations in purification conditions. Mice were kept without food for 15 to 20 hours and then killed by decapitation. Their livers were excised and washed once with ice-cold homogenization buffer [20 m*M* Tris-HCl (pH 7.4), 0.1 M NH_4Cl, 5 m*M* $Mg(CH_3COO)_2$, 2 m*M* 2-mercaptoethanol, 0.2 M sucrose]. All subsequent procedures were carried out at 0 to 4 °C. The livers were minced with scissors and homogenized in 2 to 3 volumes of homogenization buffer in a motor-driven Teflon-glass homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant was recentrifuged at 100,000 *g* for 2 hours.

The supernatant was brought to 25% saturation of ammonium sulfate with stirring and centrifuged at 10,000 *g* for 30 min. Further ammonium sulfate was added to the supernatant to 65% saturation and the precipitate obtained by centrifugation was dissolved in buffer G [20 m*M* Tris-HCl (pH 7.5), 0.1 m*M* EDTA, 10 m*M* 2-mercaptoethanol, 0.1 M NH_4Cl, 10% glycerol] and dialyzed against the same buffer. The dialysate was then applied to a column of Sephadex G-200 (1 × 90 cm) previously equilibrated with buffer G. The column was developed with buffer G at a flow rate of 5 ml/hr and 1 ml-fractions were collected. Active fractions were pooled and dialyzed against buffer P [50 m*M* Tris-HCl (pH 7.9), 0.1 m*M* EDTA, 2 m*M* 2-mercaptoethanol, 10% glycerol] containing 25 m*M* KCl, and then applied to a 1.4 × 12 cm column of phosphocellulose (Whatman P-11) equilibrated with buffer P containing 25 m*M* KCl. The column was washed with the same buffer containing 25 m*M* KCl, and EF-2 was eluted with 120 ml of a gradient from 25 to 400 m*M* KCl in buffer P. Fractions of 2 ml were collected, and active fractions were dialyzed against buffer H [5 m*M* potassium phosphate (pH 7.2), 25 m*M* KCl, 2 m*M* 2-mercaptoethanol, 10% glycerol]. The preparation of partially purified factor was divided into small aliquots and stored at −80 °C until use.

EF-2 of rat liver was prepared similarly except that, after ammonium sulfate fractionation, a hydroxylapatite column was used instead of a Sephadex G-200 column to separate EF-1 and EF-2. The column (1.4 × 17 cm) was developed with 200 ml of a linear gradient of 5 to 400 m*M* potassium phosphate, and fractions containing EF-2 were purified further by phosphocellulose column chromatography, as described above.

Preparations of tRNA and [^{3}H]-Phe-tRNA

Unfractionated tRNA was prepared from rat liver by the method used by Nishimura (1971) with slight modifications and labeled as follows. The reaction mixture (3 ml) consisted of 50 m*M* Tris-HCl buffer (pH 7.4), 50 m*M* KCl, 10 m*M* $MgCl_2$, 5 m*M* ATP, 25 μCi of [^{3}H]-phenylalanine, 600 μg of unfractionated tRNA, and 320 μg protein of rabbit reticulocyte S-100 obtained by 30–60% saturated ammonium sulfate fractionation. The mixture was incubated for 45 min at 37 °C, chilled in an ice bath, and adjusted to pH 5 with 3 M acetic acid. The resulting mixture was then shaken with an equal volume of water-saturated phenol and centrifuged at 3,000 *g* for 15 min at room temperature. The aqueous phase was dialyzed against distilled water, and concentrated by lyophilization. The dried material containing [^{3}H]-Phe-tRNA was dissolved in a small volume of distilled water and stored at −20 °C.

Assays of EF-1 and EF-2

EF-1 catalyzes the binding of aminoacyl tRNA to ribosomes, but its activity can be determined by assay of polypeptide synthesis dependent on EF-2. EF-2 catalyzes detachment of uncharged tRNA and translocation of peptidyl tRNA, allowing new aminoacyl tRNA to bind to ribosomes for further elongation of the peptide chain. The activity of EF-2 can, therefore, be determined by assay of polypeptide synthesis dependent on EF-1.

The activities of EF-1 and EF-2 were determined by assays of poly(U)-dependent polyphenylalanine synthesis in the presence of a saturating amount of the complementary factor (EF-2 or EF-1) obtained from rabbit reticulocytes by similar procedures to those described under "Preparation of elongation factors." The reaction mixture (30 μl) consisted of 30 m*M* HEPES/KOH buffer (pH 7.3), 8 m*M* $Mg(CH_3COO)_2$, 5 m*M* dithiothreitol, 1 m*M* ATP, 0.4 m*M* GTP, 10 m*M* creatine phosphate, 7 units/ml of creatine phosphokinase, 0.13 mg/ml of [^{3}H]-Phe-tRNA (4400 cpm/μg), 0.6 mg/ml of poly(U), 27 A_{260} units/ml of ribosomes, a saturating amount of one complementary factor (EF-1 or EF-2) of rabbit reticulocytes, and the test fraction of mouse or rat elongation factor. After incubation for 30 min at 37 °C, the reaction was stopped by adding 2 ml of ice-cold 5% trichloroacetic acid (TCA) and the mixtures were heated at 90 °C for 15 min. Insoluble materials were trapped on glass fiber filters (Whatman, GF/C), and the filters were washed with 20 ml of 5% TCA and 1 ml of 95% ethanol, and dried under an infrared lamp. The radioactivity on the filters was measured in 8 ml of toluene scintillation cocktail (4 g/l DPO, 0.1 g/l POPOP) in an Aloka liquid scintillation counter (model LSC 700).

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Heat-inactivation of EF-2

Partially purified EF-2 was heated at 50 °C for 2 to 15 min in a buffer consisting of 5 m*M* potassium phosphate (pH 7.2), 25 m*M* KCl, 2 m*M* 2-mercaptoethanol, and 10% glycerol. Then the mixtures were chilled in an ice bath and the remaining activity was determined under standard assay conditions.

To compare patterns of heat-inactivation of "young" and "old" EF-2's, amounts of the EF-2 used were such that activities before heating were nearly the same.

RESULTS AND DISCUSSION

Preparation of EF-2

The EF-1 and EF-2 used for the assay of mouse and rat factors were prepared from rabbit reticulocytes. The rabbit factors were active for poly(U)-dependent polyphenylalanine synthesis only when EF-2 and EF-1, respectively, were added to the reaction mixture (data not shown). Thus the EF-1 preparation did not contain EF-2 activity, and vice versa. EF-1 and EF-2 from mouse and rat livers in fractions of eluate from columns were located by assays in the presence of excess amounts of EF-2 and EF-1, respectively, from rabbit reticulocytes.

Figures 1a and 1b show the elution profiles of EF-1 and EF-2 from young and old mice on a Sephadex G-200 column. EF-1, with a molecular weight of more than 2×10^5, was mostly recovered in the void volume, while EF-2, with a molecular weight of about 1×10^5, was eluted later, consistent with a report by others (Bielka *et al.*, 1982). Active fractions of EF-2 were purified further by phosphocellulose column chromatography (Figure 2). Most of the protein did not bind to the column, while EF-2 from both young

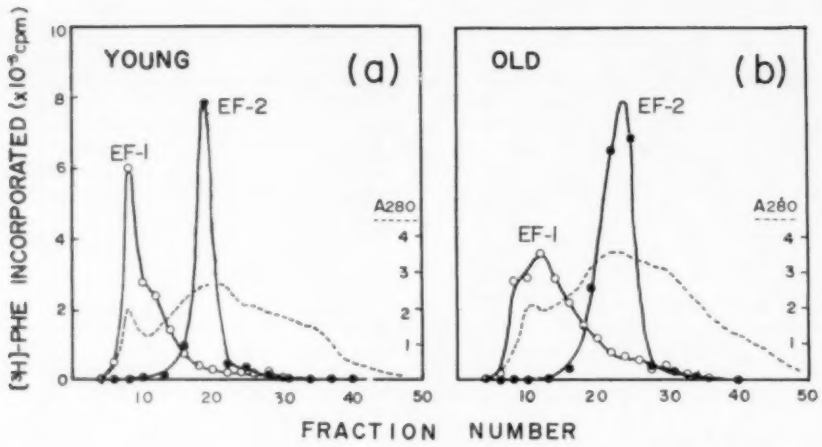

FIG. 1. Sephadex G-200 column chromatography of ammonium sulfate fractions of EF-1 (○) and EF-2 (●) from young (a) and old (b) mouse livers. Amounts of protein applied were 110 mg and 170 mg, respectively. Five μl aliquots of every other fraction diluted 10-fold were used for the assay of EF-1 and EF-2.

and old animals was eluted with about 170 m*M* KCl. The factors from young and old animals showed similar elution profiles on both the Sephadex G-200 and phosphocellulose columns. These results indicate that no gross structural alteration of either factor occurs during aging. The same was true for the factors from rat livers (data not shown).

No EF-1 activity was detectable in the EF-2 preparations from young or old mice or rats. No significant differences were observed in the specific activities of EF-2 from the livers of young and old animals, as shown in Table 1. Thus the factors of young and old animals seemed to be purified to similar extents.

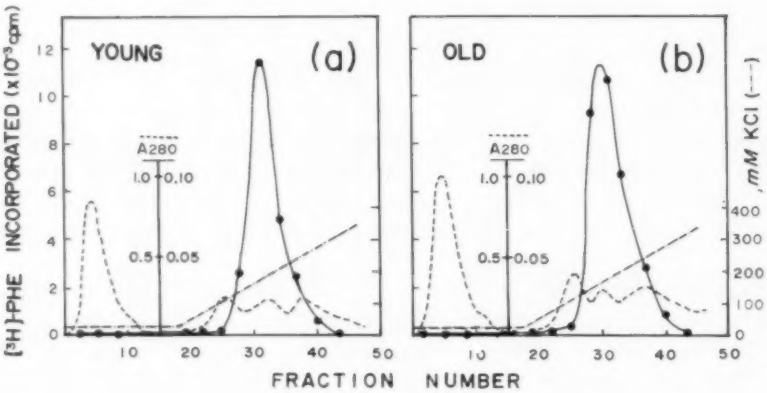

FIG. 2. Phosphocellulose column chromatography of EF-2 (●) separated by Sephadex G-200 column chromatography: (a) young and (b) old mice. Amounts of protein applied were 20 mg and 28 mg, respectively. Five μl aliquots were used for the assay of EF-2.

TABLE 1. SPECIFIC ACTIVITIES OF PARTIALLY PURIFIED EF-2 FROM YOUNG AND OLD MOUSE AND RAT LIVERS

Animal	*Age*	*Specific activity (fmol/min/μg protein)*
Mouse	Young (5 mth)	17.5
	Old (22 mth)	15.3
Rat	Young (5 mth)	14.6
	Old (19 mth)	14.6

One unit is defined as the amount of enzyme that catalyses incorporation of 1 fmol of [^{3}H]-phenylalanine into protein per min at 37 °C.

Heat-stabilities of EF-2 from young and old animals

The extent of poly(U)-dependent polyphenylalanine synthesis was linearly dependent on the time of incubation up to at least 40 min in the presence of appropriate amounts of "young" or "old" EF-2 preparation (Figure 3). To compare the activity of different EF-2 samples, the reaction was terminated at 30 min when linearity of the relation between the time of incubation and protein synthesis held well.

Figures 4a and 4b show typical thermal inactivation curves of EF-2 from the livers of mice and rats. In both cases "young" and "old" EF-2 exhibited biphasic patterns of inactivation, indicating the presence of EF-2 molecules with different heat labilities. The percentage of heat-labile components was obtained by extrapolating the slope of more stable components to zero heating time. The proportions of heat-labile components in EF-2 preparations from young and old mice were about 5 and 15%, respectively, while those in preparations from young and old rats were 10 and 35%, respectively (Table 2). Thus, the proportion of heat-labile EF-2 increased with age. However, there might be components in the partially purified preparations that affect the heat-stability. To examine this possibility, we examined the heat-stability of a mixture of equal amounts of the EF-2 preparations from young and old animals. The percentage of heat-labile components was found

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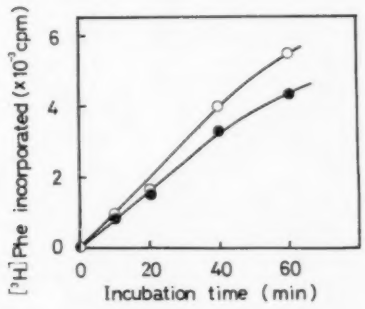

FIG. 3. Time course of polyphenylalanine synthesis dependent on poly (U) and EF-2. EF-2 was partially purified from young (5 months) (○) and (22 months) (●) mice.

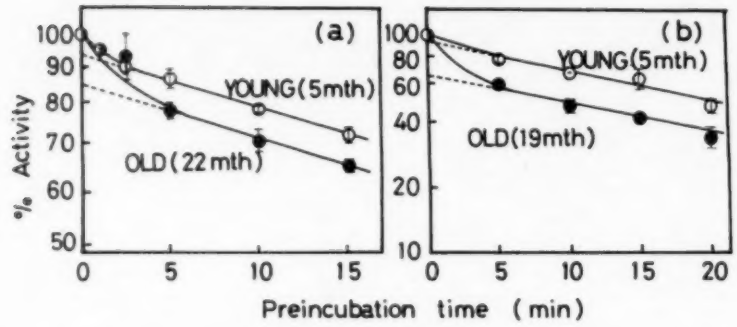

FIG. 4. Thermal inactivation curves of EF-2 from the livers of young and old mice (a) and rats (b). Partially purified EF-2 was heated at 50 °C for 2 to 20 min to buffer H. Then residual activity was determined under standard assay conditions.

to be about the average of those of the factors from young and old animals determined separately (Figure 5). Thus these results excluded the possibility that activation or inactivation factors are present in the preparations from young and old animals. So we concluded that there are more heat-labile molecules in the EF-2 preparation from old animals than in that from young ones.

The heat-lability of the elongation factor determined in vitro does not necessarily reflect the lability in vivo. But it is possible that larger proportions of the factor in old animal tissues undergo some minor structural alterations that can only be revealed by heating in vitro, and that are less active or easily inactivated in vivo, thus contributing to decrease in protein synthesis.

Various enzymes altered in properties such as heat-stability, cofactor binding, specific activity, or immunological reactivity are known to accumulate in many tissues during aging (Gershon, 1979; Rothstein, 1979). To the list of such proteins should be added the proteins of translation machinery, that is, EF-2 (this report) and aminoacyl tRNA synthetase (Takahashi *et al.*, in press).

TABLE 2. SUMMARY OF RESULTS ON HEAT-STABILITY OF EF-2

	Animal	*Age*	*Heat-labile fraction (%)*
Exp. 1	Mouse	Young (5 mth)	10
	(n=2)*	Young + Old	25
		Old (22 mth)	45
Exp. 2	Mouse	Young (5 mth)	5
	(n=2)	Old (22 mth)	15
Exp. 3	Mouse	Young (5 mth)	0
	(n=2)	Old (20 mth)	25
Exp. 4	Rat	Young (5 mth)	10
	(n=1)	Old (19 mth)	35

*Number of young or old animals used for the preparation of EF-2.

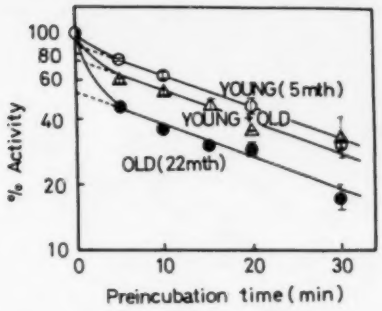

FIG. 5. Thermal inactivation curves of EF-2 of young (○) and old (●) mouse livers, and of a mixture (△) of equal amounts of "young" and "old" EF-2's. Mixtures were heated at 50 °C for 5 to 30 min in buffer H. Then residual activity was determined under standard assay conditions.

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QUANTITATIVE STUDIES OF THE EFFECTS OF AGING, MECLOFENOXATE, AND DIHYDROERGOTOXINE ON INTRANEURONAL LIPOPIGMENT ACCUMULATION IN THE RAT

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Abstract—Intraneuronal lipopigment accumulation is associated with ageing and certain diseases, and there are many claims that this can be influenced by drugs, particularly meclofenoxate (centrophenoxine). The various unsubstantiated or conflicting reports of the effects of this drug in animal studies indicate the need for methods for the demonstration of lipopigment accumulation in adequately defined, easily-identified, and relatively homogeneous neuronal populations; this study has validated two such methods by demonstrating significant differences between groups of rats at different ages in respect of measured lipopigment autofluorescence intensity from the most heavily pigmented regions of a subpopulation of Purkinje cells, and of the area overlying intraneuronal lipopigment in a region of the hippocampus. These methods were then used to investigate the effects of daily (5 days per week) intraperitoneal injections of meclofenoxate or dihydroergotoxine, over a period of 12 weeks, before sacrifice at 13.5 months. No significant effects of meclofenoxate were detected, but dihydroergotoxine administration was associated with a significant increase in mean area overlying intraneuronal lipopigment in the CA3a region of the hippocampus. The results do not confirm that meclofenoxate can induce a reduction in intraneuronal lipopigment, but suggest that chronic dihydroergotoxine administration was associated with an increase in intraneuronal volume of lipopigment in the cell bodies of CA3a hippocampal neurones.

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INTRODUCTION

IT HAS been claimed that intraneuronal lipopigment accumulation can be correlated with ageing, functional activity, diet, certain diseases, and several drugs, particularly meclofenoxate, otherwise known as centrophenoxine (Dowson, 1982a). However, despite the many claims that this drug can induce a reduction in volume, and other changes, in the lipopigment in cell bodies of various neuronal populations, the histological evidence for such effects is inconclusive.

Investigation of the effects of drugs on the volume of neuronal lipopigment requires methods for the quantification of lipopigment in easily identified and relatively homogeneous neuronal populations, and the present study has validated two methods, by demonstrating statistically significant differences between three groups of rats at different ages. Two types of neurone were examined, involving subpopulations of hippocampal neurones and of Purkinje cells. It should be noted that Heinsen (1981) reported significant differences in lipopigment volume in Purkinje cells between various regions of the cerebellum.

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Most histological quantitative studies of neuronal lipopigment have involved point-counting or similar methods, which investigated the area overlying lipopigment in tissue sections. However, this technique would be expected to be progressively less accurate as individual lipopigment granules become aggregated (Dowson and Harris, 1981), and a method for the measurement of autofluorescence intensity from regions of lipopigment has been validated as an alternative quantitative technique. (Dowson and Harris, 1981; Dowson, 1982a). The fact that marked variations in lipopigment autofluorescence intensity can be associated with regions of aggregated granules of pigment overlaid by similar areas, clearly demonstrates that measurement of autofluorescence intensity from lipopigment can provide an additional dimension of data, compared with point-counting methods (Dowson and Harris, 1981). However, although a correlation can be observed between volume of lipopigment and autofluorescence intensity, several other variables have the potential to affect this relationship, and a linear correlation between lipopigment autofluorescence intensity and volume should not be assumed (Dowson 1982a).

In the present study, a method for measurement of lipopigment autofluorescence intensity from the most heavily pigmented regions of rat Purkinje cells was developed, and was used to demonstrate significant differences between three groups of rats aged 6.5, 13.5, and 23.5 months, respectively.

The other neuronal population that was examined consisted of pyramidal neurones in a specified region of horizontal sections of rat hippocampus. In these cell bodies, lipopigment was less aggregated than in the Purkinje cells, and a variation of a point-counting method was considered to be the optimal method for the quantification of lipopigment. Differences between the three groups of rats, aged 6.5, 13.5, and 23.5 months, respectively, were also demonstrated by this technique.

Both these methods were then used to investigate the effects of chronic administration of meclofenoxate and dihydroergotoxine. The aim of the present study was to confirm previous reports that chronic meclofenoxate administration has been associated with a reduced volume of intraneuronal lipopigment, and to investigate whether dihydroergotoxine has a similar effect. In clinical practice both these drugs have been used in attempts to treat various forms of chronic cognitive impairment (Dowson, 1982b), and the effects of dihydroergotoxine on intraneuronal lipopigment are of particular interest, as, in the clinical literature, dihydroergotoxine is the most extensively studied of all the drugs used for this purpose. Although many publications have examined the effects of meclofenoxate on intraneuronal lipopigment, there is only one report involving the effect of dihydroergotoxine on lipopigment, which claimed that this drug was associated with reduced lipopigment in cultured mouse neuroblastoma cells. (Nandy and Schneider, 1978).

In the present study, the two methods that have been outlined were used to investigate the effects of intraperitoneal injections of meclofenoxate or dihydroergotoxine over a period of 12 weeks, up to 13.5 months of age. A third control group received injections of saline.

METHODS AND PROCEDURES

Female Wistar rats were housed in conventional facilities with ad libitum access to water and a commercial pellet diet. Groups of rats were sacrificed by exposure to chloroform vapour after 6.5, 13.5, and 23.5 months, respectively, and the brains were stored in 10% formalin for up to 17 months before processing. (This variable is unlikely to have had more than a minimal effect on the results, as the measured fluorescence intensity correlated well with observed estimations of the marked intergroup differences in pigment volume). Three additional groups received intraperitoneal injections of either meclofenoxate, dihydroergotoxine, or saline, and for each

group a total of 60 injections were given on each of 5 days per week over a period of 12 weeks. The animals were sacrificed at the age of 13.5 months. For these groups, the time of storage of brain tissue in formalin was constant.

Meclofenoxate hydrochloride was supplied as Helfergin by Promonta (Hamburg, Federal Republic of Germany), and a solution, in the medium provided (containing Poly-1-vinyl-2-pyrrolidon), was prepared within 30 minutes of injection at a dose of 80 mg/kg in approximately 0.6 ml. (It should be noted that this medium induces a sterile peritonitis; saline is an alternative solution medium, which, however, may not provide equivalent stability of the compound). Dihydroergotoxine was supplied as Hydergine by Sandoz Products Ltd (Leeds, U.K.), and a fresh solution was prepared within 30 minutes of injection by dissolving 5 mg in 1 ml of ethanol, which was then diluted with saline to an 8% ethanol solution. A dose of 1 mg/kg was then administered in approximately 1 ml.

Two subpopulations of Purkinje cells were examined. In the comparison of the three groups of rats of different ages, each cerebellum was sectioned in the mid-sagittal plane, and 20 μm sections from the left side were studied within 100 μm of the plane of dissection.

Purkinje cells were examined within lobule I of the vermis according to the terminology of Larsell (1952), whereas in the investigation of the effects of the drugs, Purkinje cells were selected within the lateral half of the left side of Crus I, after the cerebellum had been divided in the transverse plane through the midpoint of the vermis at the declival sulcus I (Larsell, 1952); 20 μm sections were examined within 100 μm rostral to the plane of dissection.

Unstained 20 μm sections were examined by epi-illumination with an Leitz Ortholux II microscope (E. Leitz, Ltd. Luton, U.K.) with photometric attachments (Dowson and Harris, 1981). The exciting light from a mercury arc lamp passed through an exciter filter that allowed the passage of violet and blue light, and the resulting autofluorescence above 580 nm was transmitted by the barrier filter (K580, Leitz) before measurement. The Purkinje cells were then examined with an X100 oil-immersion objective (NA 1.32). A graduated line was superimposed upon the row of Purkinje cells in the field, and a position on the line was randomly selected. The Purkinje cell body that was nearest to this point was then selected for measurement if it could be safely identified as a Purkinje cell and had a minimum dimension, in any direction within the plane of the section, of 15 μm.

The cell was then positioned so that the fluorescence intensity from its most heavily pigmented region, delineated by a circle with a diameter of 11 μm, could be measured. Although this procedure had the disadvantage that the observer had to judge and define the most heavily pigmented region of each cell, most cells contained a predominant aggregation of pigment that could be encompassed by the selected size of each measured region. An alternative procedure would have been to measure the fluorescence from the entire Purkinje cell body, but this would have produced an unacceptably high degree of contamination of the measured yellow lipopigment fluorescence by green background tissue autofluorescence. The time intervals between these various procedures were kept as constant as possible to minimize variation in the effects of fading of autofluorescence during irradiation. (Dowson and Harris 1981; Dowson, 1983).

On each section, this procedure was repeated so that a total of eight Purkinje cells were examined, in a series of eight adjacent fields. In lobule I the most caudal field was the first to be examined, whereas in Crus I, the position of the initial field and subsequent direction of adjacent fields was also constant. In addition, two measurements of the intensity of "background" green tissue autofluorescence were taken from equivalent regions that did not contain lipopigment; these values were subsequently used in the following correction procedure to minimize the effect of the contamination of each measurement of lipopigment fluorescence by background green fluorescence. Eight Purkinje cells from each of two adjacent sections were examined from each rat, and the mean of the four corresponding regions of background fluorescence was subtracted from the mean of the 16 measurements. The resulting value, in a constant arbitrary unit, represented the mean contribution of yellow lipopigment autofluorescence to the mean of the 16 measurements obtained from each rat. It must be remembered that an increasing volume of lipopigment displaces a corresponding volume of background autofluorescence, but, if there are approximately constant relationships between fluorescence intensity and volume, for both lipopigment and background tissue, such displacement would not influence the ratios between the various corrected values for lipopigment autofluorescence intensity that are obtained by this method. However, this correction procedure, which involves approximations, would alter the value of the corrected arbitrary unit of lipopigment fluorescence intensity if there was a change in the relationship of fluorescence intensity to volume for either lipopigment or background tissue. However, corrected values of fluorescence intensity derived from lipopigment are valid data in themselves, if interpreted in the knowledge of the potential variables other than pigment volume. Further methodological details have been reported elsewhere. (Dowson and Harris, 1981; Dowson, 1982a).

Pyramidal cells were examined from the stratum pyramidalis of horizontal sections of the left side of the hippocampus and cells were selected from the CA3a region. (de No, 1934). The plane of the sections was parallel to the horizontal zero plane (König and Klippel, 1963), and the most rostral section of the eight adjacent sections that were examined was approximately 2 mm inferior to the superior surface surface of the splenium of the corpus callosum in the mid-sagittal plane.

Unstained 8 μm sections were examined with the Leitz Ortholux II microscope as described in a previous paragraph except that a barrier filter was used that transmitted above 515 nm. A X63 oil-immersion objective (NA

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1.3) enabled each CA3a region to be divided into four adjacent fields, and one hippocampal neurone was selected from each field by superimposing a 20-intersection grid, selecting an intersection at random, and then identifying the neuronal body nearest to the chosen intersection. To be selected, a neuronal body had to be discrete and contain a visible nucleolus. The focus of the X100 oil-immersion objective was adjusted to visualize the maximum amount of lipopigment in the cell body, and the cell was then photographed (using the Wild Photoautomat MPS55 system) with 400 ASA Fujichrome colour film. Eight adjacent sections, and therefore, a total of 32 cells were examined from each rat brain.

The transparencies of the photographed cell bodies were then projected onto paper keeping the magnification constant, and the outlines that delineated areas overlying yellow lipopigment were drawn. The total area enclosed in these outlines, and the number of discrete outlines, were measured automatically for each cell with a Quantimet 720 Image Analyser (Cambridge Instruments Ltd. Cambridge, U.K.).

RESULTS

The results in Table 1 demonstrate that the method for measuring the intensity of lipopigment autofluorescence from the most heavily pigmented regions of rat Purkinje cell

TABLE 1. LIPOPIGMENT FLUORESCENCE INTENSITY DERIVED FROM THE MOST HEAVILY PIGMENTED REGIONS OF RAT PURKINJE CELL BODIES

Age (Months)	*6.5*	*13.5*	*23.5*
Number of rats	5	9	9
Mean of corrected measurements of lipopigment fluorescence intensity (Arbitrary units)	3.2 (SEM 1.1)	13.4 (SEM 0.7)	29.4 (SEM 2.2)

Each corrected measurement was the mean of 16 values from 16 Purkinje cells from each rat. Measured regions had a diameter of 11 μm.

TABLE 2. THE EFFECT OF 12 WEEKS OF ADMINISTRATION OF MECLOFENOXATE AND DIHYDROERGOTOXINE ON THE LIPOPIGMENT FLUORESCENCE INTENSITY DERIVED FROM THE MOST HEAVILY PIGMENTED REGIONS OF RAT PURKINJE CELL BODIES

Treatment	*Meclofenoxate 80 mg/kg/i.p.*	*Dihydro-ergotoxine 1mg/kg/i.p.*	*Saline i.p.*
Age	13.5	13.5	13.5
Number of rats	8	7	8
Mean of corrected measurements of lipopigment fluorescence intensity (Arbitrary units)	14.6 (SEM 2.1)	13.7 (SEM 1.5)	13.1 (SEM 1.2)

Each corrected measurement was the mean of 16 values from 16 Purkinje cells from each rat. Measured regions had a diameter of 11 μm.

TABLE 3. THE EFFECT OF AGEING ON THE AREA OVERLYING LIPOPIGMENT AND ON THE NUMBER OF DISCRETE REGIONS OF LIPOPIGMENT IN HORIZONTAL 8 μM SECTIONS OF RANDOMLY-SELECTED NUCLEOLUS-CONTAINING NEURONAL BODIES IN A REGION OF RAT HIPPOCAMPUS

Group *Age (Months)*	*A* *6.5*	*B* *13.5*	*C* *23.5*
Number of rats	5	8	9
Mean area over lipopigment per neurone (μm^2)	8.3 (SEM 1.5)	18.2 (SEM 2.3)	29.4 (SEM 2.4)
Mean number of discrete regions of lipopigment per neurone	11.9 (SEM 1.2)	13.5 (SEM 0.6)	10.4 (SEM 0.5)

Mean area: B > A, $P = 0.01$*; C > B, $P < 0.02$*
Mean number: C < B, $P < 0.02$*
*(Mann-Whitney *U* test, 2-tailed)

bodies was able to distinguish between the groups of rats at three different ages. The difference between any pair of the corrected measurements of the three groups was significant at $P < 0.02$, two-tailed (Mann-Whitney *U* test, Siegel, 1956).

This method was then used to investigate the effects of administration of meclofenoxate or dihydroergotoxine, and no significant effects on Purkinje cell lipopigment autofluorescence were detected. (Table 2). (The measurements of "background" tissue autofluorescence were not significantly different between the three groups in both Table 1 and Table 2.)

Table 3 shows the relationships between aging, the area overlying lipopigment, and the number of discrete regions of lipopigment in horizontal 8 μm sections of the CA3a region

TABLE 4. THE EFFECT OF 12 WEEKS OF ADMINISTRATION OF MECLOFENOXATE AND DIHYDROERGOTOXINE ON THE AREA OVERLYING LIPOPIGMENT AND ON THE NUMBER OF DISCRETE REGIONS OF LIPOPIGMENT IN HORIZONTAL 8 μM SECTIONS OF RANDOMLY-SELECTED NUCLEOLUS-CONTAINING NEURONAL BODIES IN A REGION OF RAT HIPPOCAMPUS

Treatment	*Meclofenoxate*	*Dihydro-ergotoxine*	*Saline (Controls)*
Age (Months)	13.5	13.5	13.5
Number of rats	7	7	8
Mean area over lipopigment per neurone (μm^2)	25.3 (SEM 3.8)	29.8 (SEM 3.4)	18.2 (SEM 2.3)
Mean number of discrete regions of lipopigment per neurone	14.1 (SEM 0.8)	16.1 (SEM 1.1)	13.5 (SEM 0.6)

Mean area: Dihydroergotoxine > Controls, $P = 0.028$ (Mann-Whitney *U* test, 2-tailed)

of the rat hippocampus; significant differences in the area overlying lipopigment were demonstrated between the three groups of rats, and the mean number of discrete areas of lipopigment per neurone was significantly less in the rats aged 23.5 months, when compared with the rats aged 13.5 months, which probably reflects an increasing aggregation with age.

Table 4 shows the effects of meclofenoxate or dihydroergotoxine on the area overlying lipopigment, and on the number of discrete regions of lipopigment, in the CA3a hippocampal region. It can be seen that both drugs were associated with an increase in the area overlying lipopigment although the mean 39% increase associated with meclofenoxate was not statistically significant at the 5% level. However, dihydroergotoxine administration was associated with a significant increase in mean area overlying lipopigment, which involved a mean increase of 64%.

DISCUSSION

It is generally believed that the chronic administration of meclofenoxate leads to a reduction in the volume of intraneuronal lipopigment in many, and perhaps all, neuronal populations, and that this is accompanied by ultrastructural changes and fragmentation of pigment aggregations. This belief is based on various reports that have involved the examination of histological sections with the light, fluorescence, and electron microscopes, and on studies involving tissue extracts or cell cultures. However, if the histological evidence concerning the effects of the drug on neurones is reviewed, only four studies have presented the statistical analysis of data. Three of these reported reduced intraneuronal pigment associated with meclofenoxate administration, whereas the remaining study found no significant changes.

Riga and Riga (1974) gave meclofenoxate in a dose of 80 mg/kg by intraperitoneal injection daily for 2 months to rats aged 24 months. A point-counting method was used in 6 μm sections, and drug-associated significant reductions in lipopigment volume were found in five out of the six cell populations that were studied. Although this was a meticulous study, there were no details of how the cells or fields were selected, and the origin of the drug and its medium for solution were not stated.

Two studies by Nandy (1978a, 1978b) reported the effects of daily intraperitoneal injections of 80 mg/kg of meclofenoxate to mice. The duration of treatment varied from 2 to 11 months and the age at sacrifice from 3 to 12 months. Cells of the frontal cortex and hippocampus were examined by a point-counting method, and significant drug-associated effects were reported. However, there were no details of the selection or location of the cells, or of many other important aspects of the methodology. It was not stated whether all the lipopigment in the sections was measured, or whether the results related only to intraneuronal pigment. Nandy claimed that a significant effect occurred after 3 months of drug treatment, but not after only 2 months.

The last of the four histological studies that provided statistical data is that of Katz and Robison (1983), who administered either 80 or 120 mg/kg of meclofenoxate intraperitoneally for 11 weeks to 25-month-old rats. Two regions were examined, namely the retinal pigment epithelium and a specified region of the frontal cortex. Lipopigment volume was estimated by the application of a point-counting method to photographs, and in the frontal cortex the combination of neuronal and extraneuronal lipopigment was studied. The method was validated by the demonstration of age-related differences in volume of lipopigment, but meclofenoxate was not associated with any significant differences from

the control group. However, it should be noted that in both cell populations the trend was in the same direction, and involved a drug-associated increase in lipopigment. This is of interest in the light of the present results.

Part of Katz and Robison's study was similar to part of Riga and Riga's work, in that both used rats about 2 years old, both used 80 mg/kg intraperitoneal doses of meclofenoxate for between 2 and 3 months, and both examined all the lipopigment in a region of frontal cerebral cortex using a point-counting method; however, contradictory results were obtained, and it has been noted that the two other studies gave insufficient details for a reliable attempt at replication to be made.

The only previous study that has used a quantitative method to examine rat Purkinje cell lipopigment is that of Riga and Riga (1974), and significant reductions in intraneuronal lipopigment were reported in Purkinje cells, although there were no details as to how the Purkinje cells were selected. It was claimed that meclofenoxate had a relatively marked effect on lipopigment with a scattered or perinuclear distribution in contrast with the effect on polar aggregations, and it is possible that such a differential effect of meclofenoxate on the pigment in Purkinje cells may not have been detected in the present study, which examined the most heavily pigmented areas. Methods for the measurement of lipopigment fluorescence intensity are probably most suitable for relatively large pigment aggregations, so if the effect of meclofenoxate is most marked on scattered, relatively non-aggregated granules, then a point-counting method may be more appropriate.

Two other studies have reported the effects of meclofenoxate on animal Purkinje cells, but their claims were not substantiated by the provision of statistically-treated data. Nandy (1968) administered 80 mg/kg for between 4 and 12 weeks to guinea pigs aged between 6 months and 2 years, and examined several regions, including the cerebellum. This study reported that the drug caused a reduction in intraneuronal lipopigment in "most parts of the CNS," which presumably included Purkinje cells, and a study by Tomonaga *et al.* (1978) involved the administration of 80 mg/kg of meclofenoxate daily, for up to 4 months, to rats aged 6 months or 2 years at the start of treatment. Although there was very little information on the methodology and no data, it was claimed that meclofenoxate produced no changes in the amount of lipopigment in neurones of the trigeminal nucleus, hypothalamus, and Purkinje cells. However, vacuolation and dispersal of lipopigment granules were said to have been observed with the electron microscope.

The present study of the chronic administration of dihydroergotoxine is the first investigation of the effects of this drug on cerebral lipopigment, and no effect on Purkinje cell lipopigment was detected. The dose of 1 mg/kg that was used is in the range of doses that have been claimed to produce various changes in the rat electroencephalogram (Carruthers-Jones *et al.*, 1978).

The present method for the study of the CA3a region of the hippocampus can be considered as equivalent to a point-counting method, although the measurements of the delineated areas were carried out automatically. One problem with the method involved the process of defining the boundary of an area of lipopigment when this was not in focus. In making this judgment, the observer designated an area as overlying lipopigment if it was judged to exhibit a definite yellow or yellow–red colour, and all the (unidentified) photographs were processed by the same observer.

Although a significant effect of meclofenoxate on the amount or dispersal of lipopigment was not found, there was a non-significant trend in the direction of a meclofenoxate-associated increase in intraneuronal lipopigment as was also reported by Katz and

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Robison (1983) in their two cell populations. The only data-containing studies that have investigated the effect of meclofenoxate on hippocampal lipopigment were by Nandy (1978a, 1978b), but these contained little information about the methodology. Both these studies claimed a drug-associated reduction in hippocampal lipopigment.

In the present study, the significant finding of a dihydroergotoxine-associated increase in mean area overlying lipopigment in hippocampal neurones suggests that this drug may have caused an increase in intraneuronal volume of lipopigment. Although each dose of the solution of dihydroergotoxine contained approximately 0.1 ml of ethanol, this would seem an unlikely cause of this result, as although it has been reported that chronic administration of 20% ethanol solution as the sole liquid source to rats was associated with increased intraneuronal lipopigment (Tavares and Paula-Barbosa, 1983) a relatively small quantity was involved in the present study. If this finding is replicated, any such effect of dihydroergotoxine may of course, be non-specific or indirect. However, it is possible that this drug stimulates neuronal metabolism and that this results in increased lipopigment accumulation, as there is evidence that volume of pigment can be correlated with previous functional activity. (Sohal, 1981). On the other hand, drug-associated lipopigment accumulation may result from a toxic action. The effects of different doses of this and other drugs will be of interest in this context.

In conclusion, many more histological studies on the effects of drugs on intraneuronal lipopigment are required and should meet three basic criteria. First, sufficient methodological details must be provided to allow replication. Second, particular attention must be paid to the definition and reasonable ease of location of each neuronal population under study, and third, statistically-analysed data must be provided. At present, no data-containing histological study on the effects of drugs on neuronal lipopigment has been reliably confirmed.

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AGE-RELATED CHROMATIN CONDENSATION IN FLIGHT MUSCLE NUCLEI OF THE ADULT MALE HOUSEFLY, *MUSCA DOMESTICA*

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Abstract—Computer analysis of indirect flight muscle nuclei from the adult male housefly, *M. domestica*, has shown significant change with age in the chromatin condensation pattern. The pattern was analyzed by examining low, medium, and high density chromatin components (LDC, MDC, HDC). No significant change occurred in HDC with age, and the amount and distribution of LDC and MDC remain relatively stable between Day 1 and Day 4 post-eclosion. However, the analysis showed a significant increase in the amount of MDC with a corresponding decrease in the amount of LDC between Day 4 and Day 14. This exchange was accompanied by a significant redistribution of both components. These results are discussed with reference to the biochemical and ultrastructural profile of the flight muscle with age, and to age-related changes in the condensation pattern of specific brain and Malpighian tubule nuclei described earlier.

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INTRODUCTION

WE RECENTLY showed an age-related increase in chromatin condensation in brain and Malpighian tubule nuclei of the adult male housefly, *Musca domestica* (Panno and Nair, 1984) and have now extended this study to include flight muscle nuclei from the adult male. Flight muscle nuclei are of particular interest because the maturation–senescence profile of this tissue appears to be different from that of the animal as a whole. Based on the activity levels of brain cholinesterase and Ca-activated ATPase (Clark and Rockstein, 1964), on mating behavior (Adams and Hintz, 1969), flight ability (Rockstein and Bhatnagar, 1966), and chromatin condensation of brain and Malpighian tubule nuclei (Panno and Nair, 1984), the male appears to be fully mature within 24 hours of adult emergence. However, Coulter-counter (Rockstein and Bhatnagar, 1965) and ultrastructural studies (Sohal *et al.*, 1972; Webb and Tribe, 1974) of the flight muscle indicate a progressive increase in the size of the mitochondria during the first week after adult emergence. This is accompanied by an increase in the activities of Mg-activated ATPase and alpha-glycerophosphate dehydrogenase during the first 4 days after adult emergency (Rockstein and Brandt, 1963).

Decline in the flight ability of the male after 4 days of age is well established (Rockstein and Bhatnagar, 1966), yet despite earlier reports of significant cytological degeneration

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during this period (Simon *et al.*, 1969; Sohal and Allison, 1971), later studies have shown that the cyto-architecture remains relatively stable with age, both in the male (Webb and Tribe, 1974; Rockstein *et al.*, 1975; also see review by Sohal, 1976) and in the female (Turturro and Shafiq, 1979).

In view of the flight muscle's unique maturation–senescence profile, we decided to examine the nuclei from this tissue and to compare any age-related change in the condensation pattern with that already established for specific brain and Malpighian tubule nuclei (Panno and Nair, 1984).

MATERIALS AND METHODS

For a detailed description of rearing conditions, tissue processing, and data analysis, see Panno (1984) and Panno and Nair (1984, in press). Indirect flight muscle from adult males was fixed in 10% buffered formalin for 2 hours, then hydrolyzed in 3.5 N HCl at 37 °C for 30 min prior to staining with Schiff's reagent. Tissue was sampled from 1-, 4-, 8-, and 14-day-old adults. Each age group consisted of five individuals, and 50 nuclei were measured from each group. In our colonies the adult male has a mean and maximum life span of 14 and 28 days, respectively.

The Schiff-stained nuclei were digitized using the Scanning Microscope Photometer (SMP, Carl Zeiss, Thornwood, NY) which is on line with PDP-12 (Digital, Marlboro, MA) and IBM-3033 computers. The nuclei were scanned using a measuring aperture of 0.5 μm diameter at a wavelength of 575 nm and a stepsize of 0.5 μm.

The analysis of digitized nuclear images involves extracting several image features from low, medium, and high density chromatin components (Panno and Nair, 1984). These components are derived from an initial boundary calculated after the methods of de Campos Vidal *et al.* (1973). The calculated boundary is an optical density that usually varies between 0.3 and 0.5 (O.D. units) depending on the tissue type and ploidy class of the nucleus, and is used to distinguish condensed from noncondensed chromatin. Differences between the various age groups, with respect to the image features, were analyzed for significance using the nonparametric Kruskal-Wallis test ($P < 0.05$), and Dunn's procedure for multiple comparisons based on rank sums (Dunn, 1964).

RESULTS

Total nuclear optical density (O.D.) and total nuclear area (expressed as the number of values in the digitized image) are shown in Figure 1A. These global features remain unchanged between Day 1 and Day 4 ($P < 0.05$) but both show a significant decrease between Day 4 and Day 14 of 10% and 22%, respectively ($P < 0.05$).

Figure 1B shows the amounts of the low, medium, and high density components (LDC, MDC, HDC) as percentages of total DNA in O.D. units, whereas Figure 1C gives the respective areas of these components as percentages of total nuclear area. Neither LDC nor MDC change significantly ($P > 0.05$) between Day 1 and Day 4. However, both components show a reciprocal change between Day 4 and Day 14 ($P < 0.05$). LDC decreases from 58% of total DNA at Day 4 to 41% by Day 14, whereas MDC increases from 29% of total DNA at Day 4 to 43% by Day 14. The amount of HDC remains at approximately the same level throughout the 14-day interval ($P > 0.05$).

Symmetry analysis (Figure 1D) shows a significant redistribution of MDC between Day 1 and Day 4 ($P < 0.05$), whereas the symmetry index of the LDC does not change over this time period ($P > 0.05$). However, the spatial distribution of both of these components changes in a reciprocal manner between Day 4 and Day 14 ($P < 0.05$). The symmetry index of HDC and the number of quadrants occupied by each component (Figure 1E) do not change significantly with age ($P > 0.05$).

Cluster analysis (Figure 1F) shows that a 1-day-old muscle nucleus has approximately two LDC and four HDC clusters, with no detectable change throughout the 14-day interval ($P > 0.05$). However, the number of MDC clusters increases between Day 1 and Day 4 ($P < 0.05$) followed by a large decrease between Day 4 and Day 14 ($P < 0.05$).

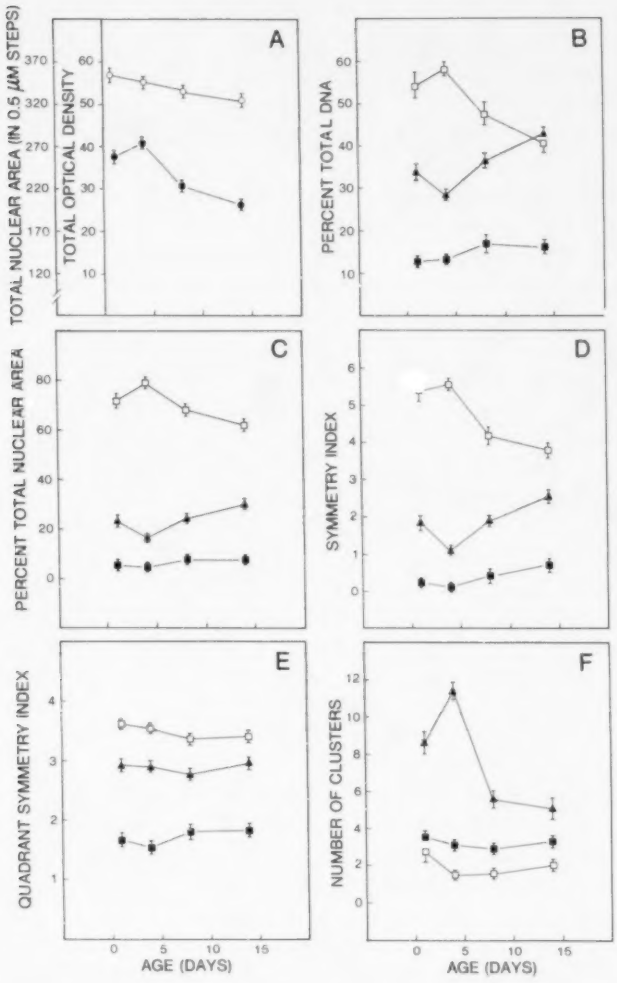

FIG. 1. Image and global features for flight muscle nuclei from adult male, *Musca domestica*. All points represent the mean ± S.E.

A: Total nuclear O.D. ○, and total nuclear Area ● . These global features show a decrease ($P < 0.05$) with age of 10% and 22%, respectively.

B: Amounts of low, medium, and high density chromatin components (LDC □, MDC ▲, HDC ■) as a percentage of total DNA.

C: Area of LDC, MDC, and HDC, as a percentage of total nuclear area.

D. Distribution of LDC, MDC, and HDC over the two-dimensional projection of the nucleus as measured by the symmetry index. Note the reciprocal exchange between LDC and MDC over the 4-day to 14-day interval in B, C, and D ($P < 0.05$), whereas HDC remains relatively stable.

E. The number of quadrants occupied by LDC, MDC, and HDC as defined by the quadrant symmetry index. This feature does not change significantly with age ($P < 0.05$).

F: The number of LDC, MDC, and HDC clusters. Note the increase in the number of MDC clusters between Day 1 and Day 4, followed by a decrease between Days 4 and 14 ($P < 0.05$), whereas the number of LDC and HDC clusters does not change with age.

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DISCUSSION

The results of pattern analysis indicate an age-related change in the condensation state of flight muscle nuclei. By dividing the total nuclear O.D. into low, medium, and high density components (LDC, MDC, HDC) we were able to show that the increase in chromatin density is due to a reciprocal exchange between LDC and MDC, whereas HDC remains unchanged. Chromatin condensation, involving an increase in the amount of MDC, affected total nuclear area, total nuclear O.D., and the precise spatial distribution of both LDC and MDC. Moreover, the increase in the number of MDC clusters between Day 1 and Day 4, and the decrease in the symmetry index of this component over the same time period suggests the possibility of chromatin decondensation during this period. Apparently the amount of LDC is increasing sufficiently during this period to separate the MDC into a larger number of clusters even though we are unable to detect a change in the amount of LDC directly. Notably there is no significant change in total nuclear O.D. or nuclear area between Days 1 and 4, although there is a trend toward increasing nuclear area during this period.

The decrease in nuclear area between Day 4 and Day 14 occurs as the chromatin becomes more compact, reducing nuclear volume and consequently total area. Chromatin condensation will also increase the resistance of the chromatin to acid hydrolysis, with a subsequent reduction in the amount of dye bound to the DNA, accounting for a 10% decrease in total nuclear O.D. between Day 4 and and Day 14. Hence, the reduction in total O.D. does not represent an age-related loss of DNA. The effect of chromatin condensation on the Feulgen reaction has been discussed by Brachet *et al.* (1968).

Our analysis of the flight muscle nuclei indicates that this tissue reaches full maturity sometime between Day 1 and Day 4, coincident with the chromatin decondensation that may occur during this period. The increase in the condensation state of these nuclei between Day 4 and Day 14 would, therefore, appear to represent the senescence phase. These results are in close agreement with the known biochemical profile of this tissue during the first 2 weeks of adulthood (Clark and Rockstein, 1964; Rockstein and Brandt, 1963). During the first 4 days after adult emergence the flight muscles are used extensively. This period of muscle development is characterized by an increase in the size of the mitochondria (Clark and Rockstein, 1964) and an increase in the activities of associated enzymes for energy metabolism (Rockstein and Brandt, 1963). Our results show that during this period the nuclei appear to remain at their lowest condensation state, although there was some indication of chromatin decondensation during this period. Between Days 4 and 14 the ultrastructural profile of the flight muscle does not appear to change significantly in the male (Rockstein *et al.*, 1975; Webb and Tribe, 1974), although the flight ability (Rockstein and Bhatnagar, 1966) and activity levels of specific mitochondrial enzymes show a significant decline (Rockstein and Brandt, 1963). Our results show that the condensation state of the nuclei increases continually throughout this period. This suggests a progressive decrease in cellular activity, based on the well documented inverse relationship between the degree of chromatin condensation and transcriptional activity (Giaretti *et al.*, 1983; Harris, 1967; Panar and Nair, 1975; also see review by Weisbrod, 1982).

Previously (Panno and Nair, 1984) we suggested that the senescence profile of an organism should not be based on the analysis of a single tissue type because physiological and biochemical data from a variety of organisms (Clark and Rockstein, 1964; Dilman and Anisimov, 1979; Finch, 1976) have indicated a nonuniformity in the rate at which different tissue types age. Moreover, we showed (Panno and Nair, 1984) that age-related

chromatin condensation in specific brain and Malpighian tubule nuclei from the adult male housefly is unique for each of these tissues. In our present study we have shown that the senescence profile of flight muscle nuclei (in terms of chromatin condensation) is notably different from both the brain and Malpighian tubule nuclei. Whereas chromatin condensation in the brain and tubule nuclei begins immediately after adult emergence, the condensation state of the muscle nuclei remains relatively stable for the first 4 days. In addition, chromatin condensation in the brain and tubule nuclei reached a plateau after 4 and 8 days, respectively, whereas chromatin condensation in the muscle nuclei occurred continually between Day 4 and Day 14. These observations support the following maturation–senescence profile for the adult male housefly: full maturation of brain and Malpighian tubules within a few hours of adult emergence followed by an immediate onset of cellular senescence, with a more prolonged maturation period in flight muscle lasting up to 4 days before the onset of senescence.

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THE QUANTITATIVE RELATIONSHIP BETWEEN LIFE SPAN, FOOD INGESTION, EGG PRODUCTION, MATING, AND FLIGHT-ACTIVITY OF PROTEIN-FED BLOWFLY *PHORMIA TERRAE NOVAE* FEMALES

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A NUMBER of investigations, especially on dipterans, have revealed the life course of the flies to be determined in a strictly ordered manner. It seems reasonable therefore to postulate a genetically determined life program (Collatz *et al.*, 1981; Collatz and Wilps, 1983; Collatz and Wilps, in press; Rockstein and Miquel, 1973).

The manifestations of this program within a given time depend, in addition to abiotic variables (such as temperature, humidity, etc.), on factors leading to successful reproduction. These factors include a sufficient food source (carbohydrates in most dipterans), the acquisition of protein-containing substrates for egg development, and the opportunity to mate.

Evidence exists that the program is under hormonal control; and reproduction, together with vegetative functions and physical activity, is integrated in a complex feedback system. According to results from Sohal *et al.* (summarized in Sohal, 1985), there exists a correlation between life span and physical activity in the housefly *Musca domestica*. Males of this species whose wings had been removed to avoid flight activity showed an increase in life span of 25% in comparison to control animals. Partridge and Farquhar (1981) showed that even sexual activity of *Drosophila melanogaster* influences the life span. Unmated males lived 65 days; when cultivated with females in a 1:1 relationship they reached a life span of only 56 days. In a male–female relationship of 1:8 the life expectancy of the male decreased to 40 days. It seems premature to generalize from these singular observations. Several contradictory results exist, at least in *Drosophila*. According to Aigaki and Ohba (1984b), virginity lengthens the female life span and shortens the male life span. "No particular relationship between egg-laying activity and female life span was proven" (p. 267).

Even based on the analysis of the activity and reproduction of single flies, they concluded that life span does not correlate directly with reproductive activity but seems to be determined by complex interactions between both sexes. In addition, the ingestion of pro-

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tein can influence the mating status via a development of ovaries. Awasthi (1976) determined that in females of the flesh fly *Sacrophaga ruficornis* ovary development took place only when these insects have access to protein. The same holds true for *Calliphora erythrocephala* (Strangways-Dixon, 1961). Among other insects a correlation between reproductive cycles and nourishment could be drawn as well. This effect of nourishment on egg development in turn could influence life span. In order to gain more insight into the energy-consuming activities during adult life and their role in determining mean and maximum life span it seemed useful to quantify metabolic and physical activities. Special attention should be paid to the investment of energy into reproduction. We propose that different modes of reproduction may be related to different aging strategies (aging with or without senescence), and attempt to evaluate this using comparative experiments (Collatz, in press; Collatz and Wilps, in press). In the following study the flight activity, amount of eggs laid, and sugar ingestion of mated and virgin females of *Phormia terrae novae* are investigated in relation to life span. Results are compared with those from females who were additionally subjected to forced physical activity for acquisition of food available only through flight activity.

MATERIALS AND METHODS

Larvae of *Phormia terrae novae* were obtained from a commercial dealer. The adults were reared under long day conditions as described elsewhere (Collatz and Hoeger, 1980). Mated populations were kept at a relationship of 1:1 between males and females.

Survival curves, flight activity, food intake, and egg production

Survival curves and flight activity were measured as described by Collatz *et al.* (1981), except that only 30 min periods of flight time were measured.

Estimation of sugar intake. Two pieces of lump sugar were weighed daily in a glass scale and placed on the bottom of the cages. On the next day, at the same time, the scales were removed and weighed again. The sugar consumption of the animals was calculated from the decrease in weight of lump sugar divided by the number of animals. In order to measure sugar intake after forced flight the glass scales were hung in the cages in such a manner that flies could reach them only by flight.

Estimation of egg production. Each day a piece of bovine heart was placed in the cages and removed the next day at the same time. The eggs were then collected and weighed. This value, along with the number of females, was used for the calculation of daily egg production. The mean value of the weight of 10–20 single flies, measured daily, was used for calculating sugar intake and egg production in mg/g fresh weight.

RESULTS

Figure 1 shows survival curves of mated and virgin females fed with protein containing substrates. The former have a mean life span (L_{50}) of 12 days and a maximum of 26 days. In contrast, virgin females have a mean life span of 20 days (L_{50}) with a maximum of 37 days. This corresponds to an increase in life span of about 40%. Although the life spans of the studied populations are shorter in comparison with earlier measurements, the ratios between the measured variables remain the same. These ratios are based on percentage of life duration for each population.

In Figure 2 the age-dependent flight performance in meters/30 minutes of both groups are shown. A drastic reduction of flight achievement is determinable in both groups as previously reported for *Phormia* males (Collatz *et al.*, 1981).

In mated females this already begins on the 12th day and come to its minimum value on the 14th day. Flight capacity in unmated females begins to decline on the 14th day and de-

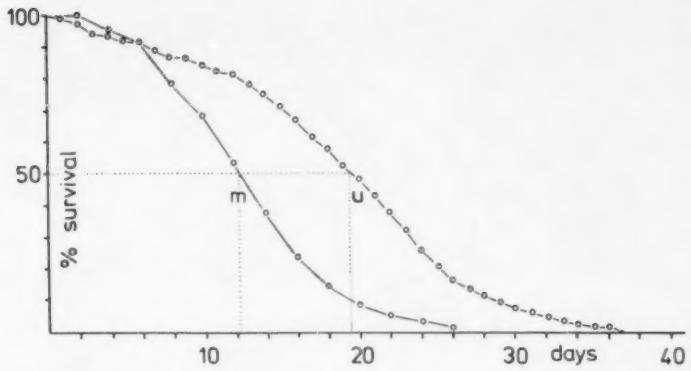

FIG. 1. Survival curves of mated (m) and unmated (u) protein provided *Phormia terrae novae* females. The single values of both curves represent a mean of 10 to 15 populations with 400 animals each, the dotted lines mark the L_{50} values.

creases gradually until a minimum is reached between the 22nd and 24th day. In addition, the absolute flight values of the virgin females are in general higher than those of the mated group.

Figure 3 shows the egg mass laid by both groups in mg/g fresh weight. The mated individuals begin with egg laying on Day 5 and reach the maximum rate on the 10th day after eclosion. In unmated animals egg deposition starts at the 10th day and has a maximum value at the 20th day. Yet the absolute mass of laid eggs in mated females is almost twice as high in comparison to the eggs of the control group. The time points of maximum egg laying also differ and correspond with the various mean life spans of both populations.

The food intake of both populations is shown in Figure 4. The highest sugar consumption of the two populations differs in the same direction as the corresponding L_{50} values and agrees with the time of maximum egg laying. Virgin females tend to ingest more sugar than mated ones, possibly due to the higher flight activity of these animals. Because the females' fresh weights after subtraction of ovary weights remain nearly unchanged during the 3 weeks after eclosion, the curves of egg production and food ingestion are the same when calculated on a per fly basis. Figure 5 shows the survival curves of mated and virgin females who acquired their carbohydrate diet only through flight activity. Both groups exhibit a shorter mean life span in comparison to the animals receiving their sugar on the bottom of the cages. This reduction of 2 days in the mated animals is smaller than that of the virgin females, which have a 4-day reduction. A curtailment of 8 days in the maximum life span is also visible in the virgin females.

Figure 6 depicts the carbohydrate intake of the populations described in Figure 5. Correlated with the reduced life span of these populations (subsequently referred to as "forced fliers"), there is an earlier maximum of sugar ingestion in comparison to the flies having their food on the ground ("ground feeders"). Further, the amount of nourishment ingestion in both groups in generally lower. The amount of food intake over the entire life span is reduced by about 30% under these conditions. Figure 7 demonstrates the egg-laying activity of the "forced fliers." Similarly, an earlier maximum of egg production in forced fliers as compared to that of the ground feeders is visible. The total number of eggs laid in

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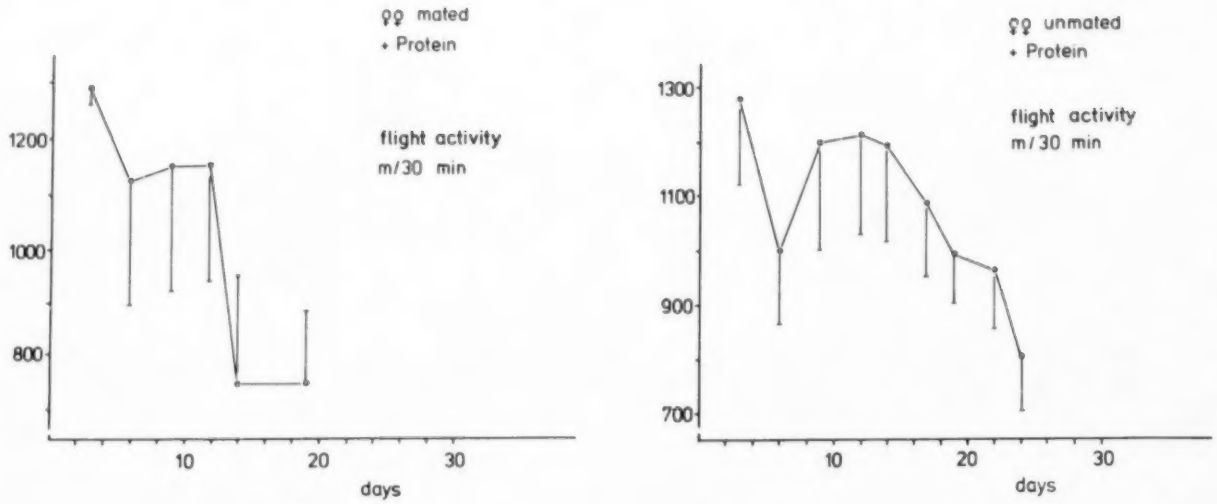

FIG. 2. Age-dependent flight activity in m/30 min of *Phormia* females. On each day the mean flight performances of 24 to 36 single flies with the corresponding standard deviations are plotted.

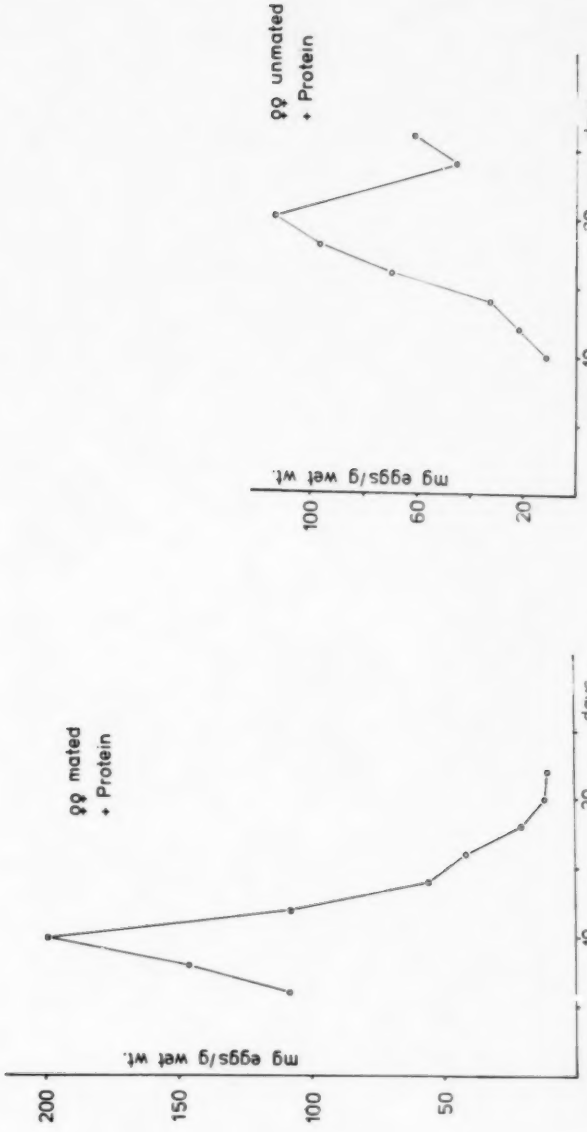

FIG. 3. Deposited egg-mass of *Phormia* females calculated in mg/g fly-wet weight. Each value represents the egg-mass of three to five populations related to the mean wet weight of 12 single flies.

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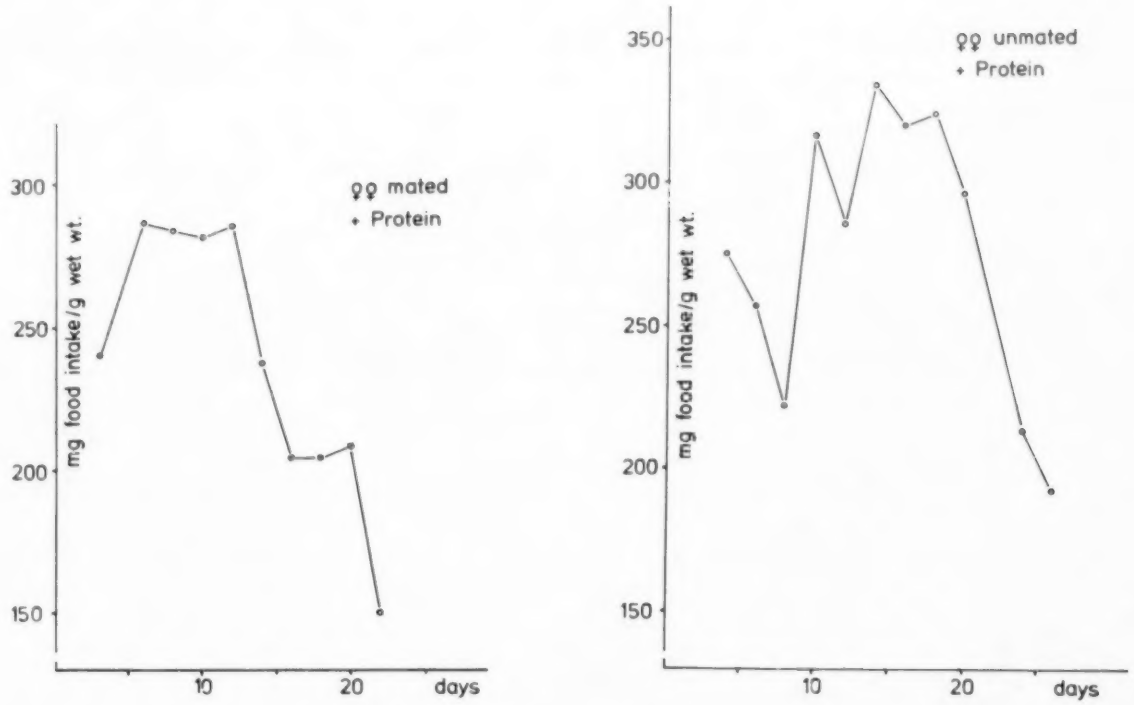

FIG. 4. Carbohydrate intake of mated and unmated protein-fed *Phormia* females. The single values represent the average of sugar intake of three to five single populations with 400 flies each. On each day food intake of the animals was measured and related to the mean wet weight of 12 single flies.

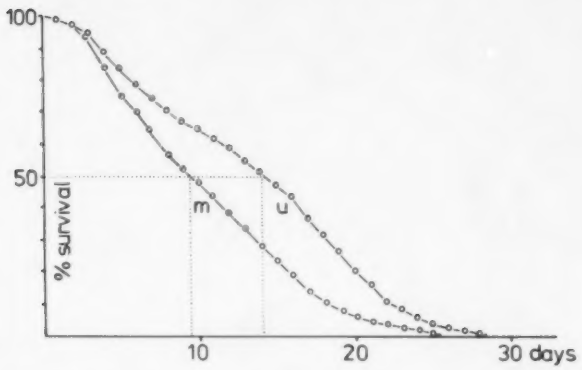

FIG. 5. Survival curves of mated and unmated protein-fed females reared as "forced fliers" (see text). The curves represent the average of three populations with 400 flies each.

both populations is less than that of the ground feeders and corresponds to the shorter average life span and the smaller intake of nourishment.

DISCUSSION

Mating reduced the mean life span of *Phormia* females by about 8 days or approximately 40% in relation to virgin animals. These findings agree well with the results of other authors (Partridge and Farquhar, 1981). However, as the careful analysis of individual flies of *Drosophila* (Aigaki and Ohba, 1984a, b) and our own results show, this reduction in life span is not simply a result of mating alone, but has to be viewed in context with the overall activities leading to successful reproduction. Unmated females show higher values of total life span, as well as total flight activity, than the comparable group of mated females. In general the lack of impairment of flight ability after a definite time after eclosion is clearly visible in a given period of 30 min. It was therefore not necessary to test the flight performance until exhaustion. If we consider the dynamics of egg production and flight performance to be evolved traits of the different insect species, then the reason for greater flight performance of unmated females could lie in the mode of reproduction of this insect. Taking into account the large number of eggs that *Phormia* is able to produce, especially in the first 2 weeks after eclosion, it seems advantageous to "invest" a considerable amount of energy in obtaining a mate even after the first unfertile eggs are deposited. This is in contrast to insects exhibiting only a moderate egg production over the whole life span. In the case of the scorpion fly *Panorpa vulgaris*, which has the latter mode of reproduction, virginity has a life-shortening effect for the females (Collatz and Wilps, 1983).

Like the mosquito *Aedes* (Lea, 1982; Masler *et al.*, 1980), ovaries of *Phormia* undergo development after eclosion. The ovary is initially in the "resting stage." Its development is stimulated by the release of juvenile hormones from the corpora allata (Masler *et al.*, 1983). Only after the intake of protein-containing substrates does the further development of the egg and its maturation take place. This process begins immediately in flies allowed to copulate after protein ingestion. In virgin females, it occurs 6–8 days later. Addition-

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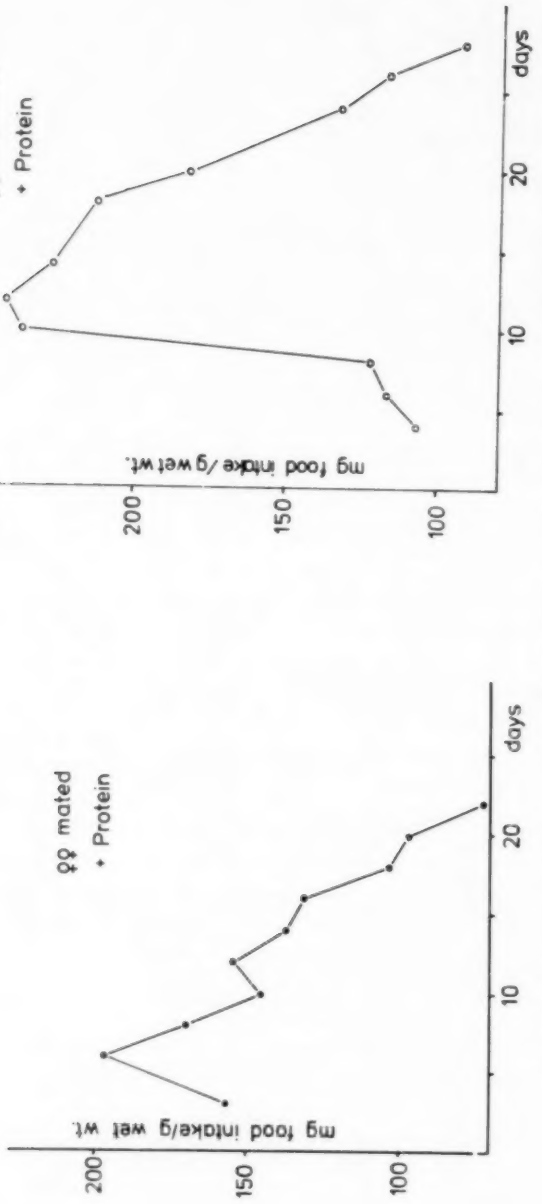

FIG. 6. Carbohydrate intake of "forced fliers." The values are the average of three populations and were calculated as described in Fig. 4.

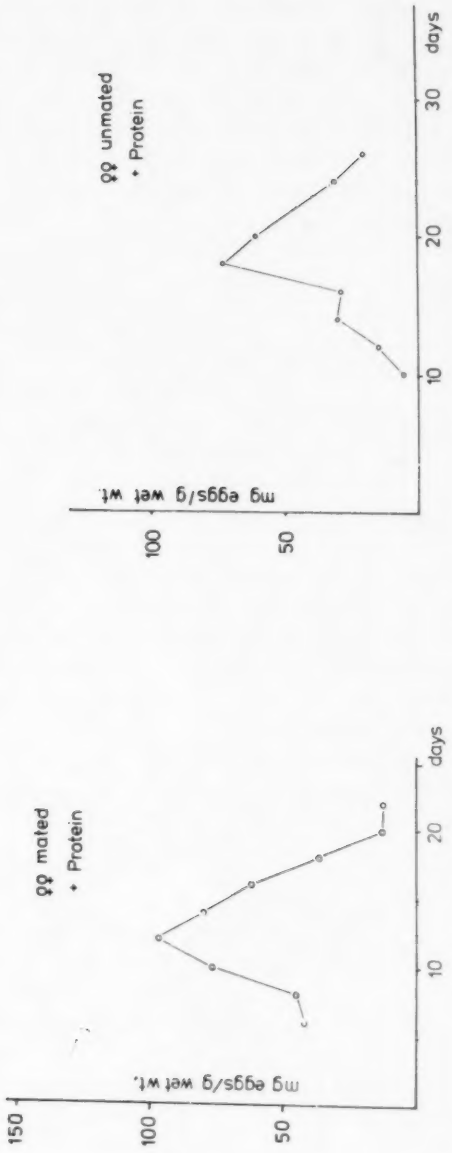

FIG. 7. Deposited egg-mass laid of "forced fliers." Calculation of the single data as in Fig. 3.

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ally, the number of eggs laid during the entire life span of virgin females comprises only 60% of those of the mated individuals. Mated as well as virgin females deposit their eggs not continuously but in clusters every 2–3 days. Together with the delayed onset of egg deposition by virgin females, this allows for the possibility of later copulation that would lead to egg fertilization. The course of flight activity during the life of mated and virgin females shows a time-dependent profile similar to that shown for the unmated male of this species (Collatz *et al.*, 1981). A phase of initial high-flight is followed by a drastic reduction in the group of mated animals on the 12th day, and in virgin females on the 16th to 18th day. Independent of the mating status, a drop in flight performance seems to occur during the first week after eclosion. This is due possibly to the first stage of ovarian development mentioned previously. The values compared with the day before and after this minimum, however, are statistically not significant.

In females allowed to copulate, the mean life span is 12 days. The maximum flight capacity remains up until this point. Between the 6th and 12th day, 80% of the total eggs of the entire life span are laid. In this manner the requirements for the existence of a new population are fulfilled before half of the predecessors have died. The following drop of flight ability in such a short time is obviously not caused by a senescent process. Preliminary results show a hormonally triggered relationship and the loss of flight performance depends on this hormonal information for reproduction activities. We therefore consider it to be an expression of a predetermined genetic program that varies as a function of time (Collatz *et al.*, 1981; Collatz and Wilps, 1983, in press). As pointed out by Collatz and Wilps (in press) the effect of the hormone action lies in a "cutting off" of the energy flow via the phosphorylase system. The details, especially the action of cAMP, adenylate cyclase, and phosphodiesterase, are under investigation.

The amount of carbohydrate ingestion is intimately coupled with the time course of egg development. A sharp increase in sugar intake by ground feeders starts initially with the commencement of egg production in both populations. Thus one can assume that the energy needed for high flight activity after eclosion and before the onset of egg production arises mainly from reserves in the body.

Despite the difficulties in extrapolating the results from laboratory studies to field conditions, it is reasonable to suppose that the observed life span characteristics of forced fliers would be more closely related to the natural situation. This is due to the fact that *Phormia*, like other dipterans, feed on flower nectar and therefore have to fly about in search of this carbohydrate source.

The data on the forced fliers show that this condition brings about a remarkable effect on the life processes, that is, reduced food ingestion, diminished egg production, and a shorter life span. Because the experimental design in this group differs from that of the other groups only in the position of the food containers, the flight activity to reach sugar was considered to be responsible for the observed variations. However, the exact amount of this obviously additional activity could not be determined. This would require the quantification of spontaneous flight activity, which perhaps may very well differ from flight performance on the flight mill.

It should be noted that in no case was the ordered pattern of activities affected by the mating status or by different holding conditions. The variations are restricted to the level of the measured parameters and the speed of the aging process. This further strengthens the theory of programmed aging in the sense mentioned by us in this paper and elsewhere (Collatz, in press; Collatz *et al.*, 1981).

SUMMARY

1. The age-dependent variations in the amount of food ingestion, egg production, and flight activity of protein-fed female *Phormia terrae novae* were analyzed. Also the influence of mating and forced physical performance on the time course of these parameters and on life span were tested.
2. In virgin females, the life span with the investigated factors is generally delayed, together with mean and maximum life span. In comparison to mated females virgin females exhibit a 40% prolongation of mean life span and ingest more sugar with a later intake maximum. Their egg production starts 5 days later (Day 10) but reaches only half the amount occurring in mated females. Flight performance is generally higher in virgin females, reaching its maximum 2 days later (Day 14), with a more gradual decline instead of the sudden drop in flight performance seen in mated flies.
3. Females receiving their food by flight only ("forced fliers") show a shorter mean life span (which is more pronounced in virgin flies). The maximum of sugar intake shifts forward to an earlier point after eclosion, and the amount is reduced by about 30% over the entire life span. Similarly, the egg production rate reaches lower values and tends to have an earlier maximum in comparison to corresponding groups that had access to sugar on the cage floor.
4. The results further support the hypothesis that *Phormia* exhibits a strictly programmed aging pattern. Its time course depends on reproduction sufficiency and physical performance. Both factors act to shorten life expectation.

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LIFE SPAN AND AGE-RELATED CHANGES IN ACTIVITY LEVEL OF THE ROTIFER *ASPLANCHNA BRIGHTWELLI*: INFLUENCE OF CURARE

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Abstract—The rotifer *Asplanchna brightwelli* shows a continuous decline in swimming activity through the course of its 5-day life span. This activity loss occurs at a slower rate when rotifers are treated with very low dosages (0.00025% to 0.0005%) curare. Rotifers treated with these low dosages of curare have a significantly longer life span than that of untreated control rotifers.

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INTRODUCTION

THE FIRST objective of this study was to determine whether there was a change in activity level of the short-lived rotifer *Asplanchna brightwelli* in the course of aging. The second objective was to determine the extent to which activity level and life span could be influenced by low levels of curare.

It has been demonstrated that the levels of spontaneous activity of rodents and insects decline with age. This age-related decline in activity has been demonstrated in rats (Slonaker, 1912; Sacher and Duffy, 1977; Hofecker *et al.* 1978; Goodrick, 1975, 1980; Strong *et al.*, 1980) and in *Drosophila* (Elens, 1972; Le Bourg, 1983). Swimming activity as a function of age has been analyzed in the rotifer *Brachionus plicatilis* by Luciani *et al.* (1983). They reported that swimming activity declines between middle and old age in this species.

An effective means of reducing or stopping activity entirely would be curare administration. The mode of action of curare is well known; it is a muscle relaxant that acts by binding to acetylcholine receptor sites (Csaky, 1979). Cholinergic neurons are present in many invertebrates (Gershenfeld, 1973); the presence of acetylcholine and acetylcholine esterase has been demonstrated in rotifers (Nogrady and Alai, 1983). It could readily be predicted that curare would reduce the activity level of rotifers, but whether curare would influence life span was not at all clear. According to the rate of living theory of aging (Pearl, 1928) agents that retard activity might be expected to extend life span. In this context, we set out to investigate whether or not low dosages of curare would influence the life span of rotifers by slowing their activity level. We began by developing a simplified

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method for activity measurement in the rotifer and by examining changes in activity level as the rotifers aged.

The questions that we investigated were as follows: (a) Does the normal activity level of rotifers change with age? (b) Would curare reduce the activity level of the rotifers? and (c) Would curare influence the life span of the rotifers?

MATERIALS AND METHODS

Culture methods

Rotifers of the species *Asplanchna brightwelli*, clone 4B61 originally obtained from Dr. John Gilbert, Dartmouth College, Hanover, New Hampshire, were used in this study. The average life span of this species is 4 to 5 days. The rotifers were maintained in a Cerophyll medium innoculated with *E. coli* and *Paramecium caudatum*, which served as their food source. The preparation of the medium was described in an earlier paper (Enesco and Verdone-Smith, 1980).

Rotifers of known birth date were individually segregated into each of the wells of 24-well tissue cultures cluster dishes (No. 76-063-05, Flow Laboratories, McLean, Virginia). The 2 ml supply of medium was replenished every 24 hours by removing 1.0 ml of old medium and adding 1.0 ml of fresh medium containing *Paramecium*. This was necessary to ensure a continuous food supply for the rotifers. The rotifers were maintained at room temperature (21°C) in continuous light conditions. Life span determinations were carried out as described in an earlier paper (Sawada and Enesco, 1984a).

Curare treatment

Curare in powder form was obtained from Brickman Chemical Co. (Montreal, Quebec) and made up as a 1% stock solution dissolved in distilled water. It was then diluted in the culture medium to concentrations of 0.0001% to 0.1% curare so that its effect on rotifer life span and movement could be tested. New medium that contained the appropriate dilutions of newly prepared curare was added daily. There were 24 rotifers in each experimental group. Treatment with curare started on Day 1 of the rotifers' life.

Monitoring of rotifer movement

To compare the movement of control and drug-treated rotifers, individual rotifers were maintained as usual in the 2.5 ml chamber of a tissue culture cluster dish. The chamber was placed on a transparent plastic sheet on which squares representing a 0.5 mm grid network had been drawn. Each rotifer could then be observed under the low power of a dissecting zoom microscope (American Optical) traversing from one square of the grid to the next. Its movement per unit time could be scored in much the same way that animal psychologists score the movement of rats or mice on an open-field checkerboard grid (Solyom, Enesco and Beaulieu, 1968). The movement count was determined by observing and recording the number of squares that the rotifer traversed in 1 minute. The results shown here represent average movement counts for groups of 24 rotifers in each treatment group.

Statistical analysis

To determine whether there were statistically significant differences between groups, a one-way analysis of variance (ANOVA) was used, followed by the *post hoc* Tukey test. These methods are described by Sokal and Rohlf (1981).

RESULTS

The results of the study on movement patterns showed that young rotifers have a much higher level of activity than old rotifers. The data in Table 1 show that the mean movement count of a 2-day-old rotifer was 67.2 squares per minute. In contrast, the mean movement count for a 5-day-old control rotifer was only 4.4 squares per minute. The steady decrease in normal activity level of control rotifers with age is graphically illustrated in Figure 1.

TABLE 1. THE EFFECT OF CURARE ON THE MEAN MOVEMENT COUNT, REPRESENTING THE AVERAGE NUMBER OF SQUARES ENTERED PER MINUTE BY ROTIFERS AT EACH AGE AND TREATMENT GROUP (N = 24). THE TOTAL OF THESE COUNTS IS USED AS AN ARBITRARY INDEX LIFETIME ENERGY EXPENDITURE

Conc. curare	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Total*
Control	67.2 ± 5.7	51.5 ± 5.5	30.0 ± 6.5	4.4 ± 3.1	0.0	153.1
0.001%	15.5 ± 4.7*	11.9 ± 4.1*	8.9 ± 3.7*	7.5 ± 3.2*	0.2 ± 0.2	43.1
0.0005%	48.2 ± 3.5*	41.3 ± 3.9	30.4 ± 3.8	23.0 ± 4.0*	16.9 ± 4.3	159.8
0.00025%	44.9 ± 3.9*	42.8 ± 3.9*	34.8 ± 4.7	24.9 ± 4.8*	7.9 ± 3.2	155.3
0.0001%	63.2 ± 3.5	53.6 ± 2.3	47.2 ± 4.1*	25.9 ± 4.7*	12.9 ± 4.0	202.8

*Significantly different from control at $P < 0.05$

Table 1 shows that the administration of curare at high concentration (0.001%) produced a dramatic decrease in the activity of 2-, 3- and 4-day-old rotifers. For example, on Day 2 the movement count was 15.5 squares per minute for the curare-treated rotifers as compared to 67.2 squares per minute for the control rotifers.

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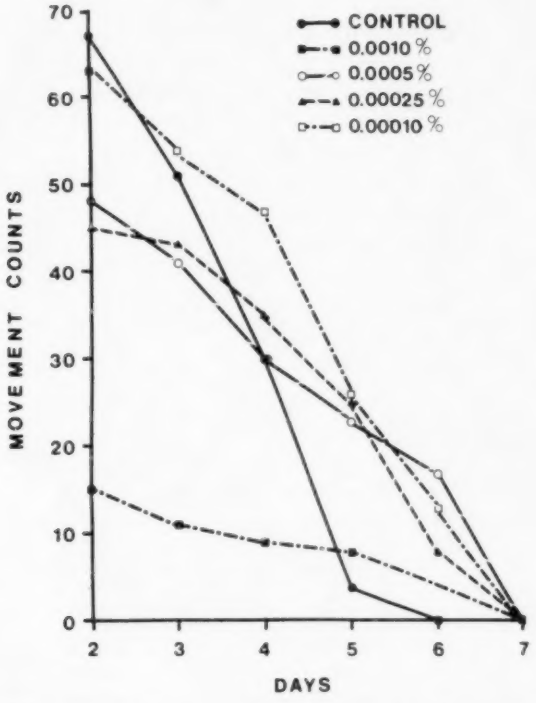

FIG. 1. The movement counts of rotifers treated with varying concentrations of curare is plotted against life span from Day 2 to Day 7. The mean movement count is the average number of squares entered per minute, and represents the average for 24 rotifers.

As the dosage of curare was steadily decreased from 0.0005% to 0.0001%, the inhibition of movement in young rotifers was gradually lifted. Table 1 shows that at the lowest concentration of curare (0.0001%) there was no statistically significant difference in the activity level of curare-treated versus control rotifers on either Day 2 or Day 3 of life.

The most striking observation in rotifers treated with the three lowest dosages of curare was that they maintained a high level of activity toward the end of their life span. Table 1 shows that on Day 5 the curare-treated rotifers had a movement count of around 25 squares per minute, whereas the control rotifers entered only 4.4 squares per minute. Thus, activity level did not fall off as rapidly for animals treated with curare as it did for the control rotifers. By Day 6, when all control rotifers had died, the curare-treated rotifers displayed a higher rate of movement than had been displayed by the control rotifers on Day 5. By adding up the activity level displayed on each day by each treatment group, one arrives at a very arbitrary estimate of total lifetime energy expenditure. It is interesting that this number is around 155 both for control rotifers and for rotifers treated with two non-toxic dosages of curare. This value is only exceeded by rotifers treated with the lowest dose of curare (0.0001%).

In Figure 1, where activity level is plotted against age, we note that there is an age-related decline in activity level for both control rotifers and curare-treated rotifers. The rate of decline in activity, indicated by the slope of the line, is the most pronounced in the control rotifers. At the highest concentration of curare used (0.001%), the initial activity level is so greatly reduced that the age-related decline in activity is very gradual. At the lower dosages of curare, the age-related decline in activity level shown in Figure 1 is much more gradual in the curare-treated rotifers than that of the control rotifers. This graphic presentation emphasizes that although curare at intermediate concentration slows the activity of rotifers early in their life span, it retards the decline of activity seen in control rotifers toward the end of their life span. At the very lowest concentration of curare, there is no inhibition of movement early in life, and the age-related decline in activity occurs at a much slower rate than in the controls. Although we had the visual impression that curare slowed the movement of the paramecia to a very slight degree, we were unable to quantitate this observation. Curare had no major deleterious effect on the paramecia.

The results presented in Table 2 show that curare treatment also had a pronounced effect on the life span of the rotifers. It should be noted that the highest concentration of curare used (0.001%) had an observable deleterious effect. Rotifers treated with this con-

TABLE 2. THE EFFECT OF CURARE ON LIFE SPAN OF *A. brightwelli* ($N = 24$)

Conc. curare	*Mean life span ± S.E.*	*% Difference from control*
Control	4.38 ± 0.22	
0.001%	3.29 ± 0.40*	−24%
0.0005%	5.79 ± 0.33*	+32%
0.00025%	5.54 ± 0.28*	+26%
0.0001%	5.88 ± 0.20*	+34%

*Significantly different from control at $P < 0.05$

TABLE 3. THE EFFECT OF CURARE ON THE REPRODUCTION OF *A. brightwelli* ($N = 24$)

Conc. curare	*Average number offspring per rotifer ± S.E.*	*% Difference from control*	*Average start of reproductive period ± S.E. (days)*	*Average end of reproductive period ± S.E. (days)*
Control	3.04 ± 0.39		2.54 ± 0.25	3.21 ± 0.35
0.001%	0.71 ± 0.31*	−77%	1.00 ± 0.37*	1.33 ± 0.49*
0.0005%	4.60 ± 0.49*	+51%	3.04 ± 0.26	4.79 ± 0.41*
0.00025%	4.10 ± 0.52*	+35%	3.12 ± 0.28	4.50 ± 0.39*
0.00010%	6.00 ± 0.36*	+97%	3.46 ± 0.12	5.42 ± 0.17*

*Significantly different from control at $P < 0.05$

centration of curare had significantly shorter life span and a significantly smaller number of offspring (Table 3) than did the controls. This is indicative of a toxic effect of curare at higher concentrations. In contrast, rotifers treated with low concentrations of curare (0.0005, 0.00025 and 0.0001%) had a significantly longer life span than that of control rotifers. In fact, the life span of rotifers with 0.0001% curare was 34% longer than that of control rotifers.

The survival curves of the curare-treated rotifers and control rotifers are presented in Figure 2. This graphic presentation emphasizes that the highest concentration of curare used (0.001%) was toxic, as shown by the sharp drop in the survival curve on the first day. In contrast, the survival curves of the rotifers treated with lower concentrations of curare are shifted to the right, indicative of life span extension.

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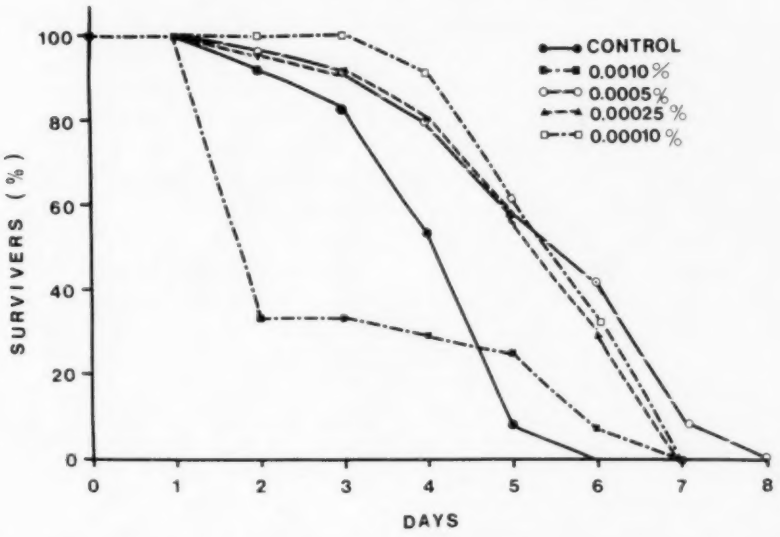

FIG. 2. The survival curves of rotifers treated with various concentrations of curare is compared with the survival curve of control rotifers.

The data in Table 3 show that rotifers treated with the lower dosages of curare had significantly more offspring than did the controls. The onset of the reproductive period of the curare-treated rotifers was later than that of the control rotifers, and extended for a longer period.

DISCUSSION

The results presented here show that there is steady decline in the activity level of rotifers as they undergo normal aging. This is in basic agreement with other studies on insects and rotifers. It is well documented that activity level of insects declines with age. For example, there is a decline in wing-beat frequency with age in *Drosophila* (Williams *et al.*, 1943) and in the house fly (Rockstein and Bhatnager, 1966). An age-related decline in flight performance has been described in mosquitoes as well (Rowley and Graham, 1968). A decline in the spontaneous activity level of *Drosophila* with age has been described by Elens (1972) and by Le Bourg (1983). The swimming behavior of rotifers has recently been analyzed by a computer-assisted automatic tracking system designed by Coulon *et al.* (1983). Using this device, Luciani *et al.* (1983) report that middle-aged females of the rotifer species *Brachionus plicatilis* swim faster than young or old females. Old animals were found to change direction less frequently and to remain closer to their point of origin.

In view of the fact that curare is a muscle relaxant and interferes with acetylcholine receptor sites (Czaky, 1979) it is not surprising that curare should retard the movement of rotifers at high and intermediate dosages or that it should have a toxic effect at high dosages. The most surprising result reported here is the finding that low dosages of curare both alter the time course of the age-related decline in activity level and significantly extend the life span of the rotifer. It is important to examine the mechanisms by which curare could bring about these changes.

One possible explanation for the increased life span in curare-treated rotifers is that the rotifers are feeding less, and are subject to dietary restriction. We have demonstrated in earlier studies that dietary restriction extends life span in this species (Verdone-Smith and Enesco 1982; Sawada and Enesco, 1984b). *A. brightwelli* is a predatory species. Clement *et al.* (1983) have shown that rotifers detect their prey by touch, and have no recognition of form or movement. Even if the movement of the rotifer and the paramecia is slowed by curare treatment, they should still come into contact in the culture well. To determine whether the paramecia were present in sufficient quantity as a food source, we examined the ingestion of neutral red-stained *Paramecia* by curare-treated rotifers using the method of Sawada and Enesco (1984b). Because we were unable to detect any difference in the density of the neutral red staining of the gut of curare treated versus control rotifers (Bozovic and Enesco, unpublished data), it seems unlikely that dietary restriction is the explanation for the curare-mediated life span extension reported here.

Another interpretation that could explain why curare prolongs life span of the rotifers could be based on the rate of living theory of aging. According to the "rate of living" theory of aging (Pearl, 1928), there is an inverse relationship between life span and metabolic rate. This theory derives from the studies by Loeb and Northrup (1917), which show that *Drosophila* reared at higher temperatures have a shorter life span than those reared at lower temperatures.

According to this theory, a poikilothermic organism has a fixed amount of life energy that it can expend, and the rate at which this energy is expended determines life span. If energy expenditure is reduced by lowering the temperature or impeding movement, life span will be increased. In tests of the "rate of living" theory of aging on poikilotherms, lowering of temperature has been the most commonly used means of lowering metabolic rate and at the same time reducing activity level (Sohal, 1982). When activity level of flies was lowered by other means, life span was also extended; these means included alteration of the sex ratio of flies (Buchan and Sohal, 1981), reduction of the flight potential of flies (Ragland and Sohal, 1973), and altering the volume of housing containers. Sohal (1984, p. 121) concludes that "life spans of flies are longer under regimens which reduce the level of physical activity."

When we examine the arbitrary total movement counts for control rotifers as compared to rotifers treated with 0.0005% and 0.00025% curare, the results are consistent with the "rate of living" theory. Although the total of the movement counts was nearly the same in all three groups, the curare-treated rotifers moved more slowly over a longer life span.

This explanation breaks down when one examines the rotifers treated with the lowest dosage of curare (0.0001%). Here, the low dosage of curare produced an arbitrary total movement count about 25% higher than that of any other group. The initial activity level of this group is not significantly different from the control on Day 2 or Day 3, and declined more slowly than the control thereafter. It appears that this very low dosage of curare has actually stimulated activity as well as increased life span in this group.

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As Sacher and Duffy (1977) point out, one must be careful to distinguish between life-prolongation that arises from a drug whose proper action (or chemical mode of action) is understood, and life prolongation that arises from hormetic action. Hormesis or hormetic action occurs when small quantities of a depressive or toxic agent produces a stimulatory effect that is qualitatively different from the effect produced over a wide range of higher dosage. Examples of hormetic effects are growth stimulation by low dosages of antibiotics or insecticides (Towsend and Luckey, 1960).

The pharmacological action of curare is understood. It slows movement in a dose-related and expected manner in this study at dosages of 0.001 to 0.00025%. We are unable to exclude the possibility that a hormetic action of curare may be responsible for the increased total movement seen when rotifers are treated with the very low dosage of 0.0001% curare.

Another factor that might influence curare action is the age of the rotifer. The 0.0001% curare had no effect on rotifer movement patterns until Day 4 of life. This suggests the rotifer becomes more drug susceptible in the course of its life span. The idea that "rate of living" is influenced by phases of the life cycle is not new. Clarke and Maynard-Smith (1961) pointed out that *Drosophila* have different responses to temperature shifts depending on their age, and proposed modifications of the rate of living theory to explain their results. The idea that the rotifer has different drug susceptibility at different phases in its life cycle could be tested by the addition of curare at differing times in the life cycle.

The simplest hypothesis to explain why intermediate dosages of curare extend life span and reduce activity is the "rate of living" theory of aging. At the very lowest dosages of curare, life span appeared to be extended without an overall reduction in activity. Complicating factors of hormetic drug action or age-dependent drug action may explain these results.

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PUBLICATIONS OF INTEREST

B.B. BOGOMOLETS

Molecular Biology of Aging

A.D. Woodhead, A.D. Blackett, and A. Hollaender, Editors. New York: Plenum Press, 1985, 482 pages. ISBN 0-306-42084-8.

Proceedings of a Symposium on the Molecular Basis of Aging, dedicated to Dr. Alex Comfort and held September 30–October 3, 1984 at Brookhaven National Laboratory, Upton, New York. A useful overview of the field by some of its principle actors. Chapters range from the 60 page article by Cutler on antioxidants to the 6 page contribution by Williams and colleagues on the responses of cultured cells to DNA-damaging agents. There is a stimulating, witty overview of the field by Comfort, a chatty piece by Lewis Thomas, and a thoughtful speculation by Holliday on the possible significance of DNA methylation in cellular aging. Major portions of the book are devoted to several chapters on Changes in DNA with Age, Aging in Cellular Proteins, and Diseases Featuring Altered Rates of Aging.

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Comparative Pathobiology of Major Age-Related Diseases: Current Status and Research Frontiers, Volume 4. Modern Aging Research

D.G. Scarpelli and G. Migaki, Editors. New York: Alan R. Liss, Inc., 1984, 441 pages. ISBN 0-8451-2304-1, LC 83-25570.

Another in the continuing stream of publications by diverse groups (in this case the Registry of Comparative Pathology) who believe that there still is ". . . a developing need not only to survey the state of knowledge regarding the aging process but also to assess the needs of the future. . . ." The alleged twist here is that emphasis is placed on ". . . the problems, challenges and opportunities for longevity studies in experimental animals." The book is composed of the collected papers of a symposium held at the NIH on 25–26 April, 1983. Chapters are grouped within headings that cover "Basic Aspects of Aging," "The Mesenchyme and Aging," and the "Aging Brain." Despite good intentions, there is precious little information on the main theme of the conference—use of animals in aging. In one of the chapters Bell perpetuates the myth that immortal cultured "normal" cells exist. He makes reference to a population of rat cells that he characterizes as normal yet the author

says that the cells transformed. Bell provides no evidence whatsoever that the endothelial cells that he studies are normal, although he misleadingly infers that they are in the context of his discussion. The fact is that no one has been able to keep a genuinely normal vertebrate cell population in a state of continuous proliferation for more than, say, 18 months.

Biomedical Basis of Gerontology

David A. Hall. Littleton, MA: PSG Publishing Co., Inc., 1984, 248 pages. $34.50.

A comprehensive survey of the aging process that spans biochemistry, demography, clinical medicine, genetics, physiology and tissue mechanics. Hall's enormous experience, lucid writing style and the numerous illustrations combine to make this one of the more important books on aging by a single author.

Aging 2000: Our Health Care Destiny, Volume 1. Biomedical Issues

C.N. Gaitz and R. Samorajski, Editors. New York: Springer-Verlag, 1985, 545 pages. ISBN 3-540-96057-0.

Proceedings of a symposium held 23–26 October, 1983 in Houston, Texas, and sponsored by the Texas Research Institute of Mental Sciences. Future planning and directions was the theme. Crystal balls were rubbed, mystical chants uttered, and visions recorded. The soothsayers covered: Basic Mechanisms of Biologic Aging, Neurochemistry of Aging and Age-Related Diseases, Imaging Techniques, Experimental Neuropathology, Prevention and Treatment of Physical Disorders, Interface of Psychiatry and Medicine, Pharmacology of Mental Illness in the Elderly, Hydergine ("A large grant by Sandoz was the vehicle that made it (the conference) possible. . . ."), and Psychiatric Treatment in the Future. A well-done overview by 64 contributors, some of whom are well-known in gerontology and others who have lofty aspirations. None have credentials as prophets.

CRC Handbook of Methods for Oxygen Radical Research

R.A. Greenwald, Editor. Boca Raton, FL: CRC Press, 1985, 464 pages. ISBN 0-8493-2936-1, $99.00.

A "How to" book for those interested in preparative and analytical methods in the oxygen radical field. Sources of reagents and instruments are given as well as descriptions of high purity sample preparation, calculations of results, and common pitfalls. The major topics include preparation of oxygen radical scavengers, oxygen radical generating systems, methods for detecting superoxide anion, hydroxy radical and singlet oxygen, measuring superoxide dismutase, methods for cellular generation of radicals and for detecting cellular damage. For free-radical theory mavens this one is a must. Others need not apply.

CRC Handbook of Nutrition in the Aged

R.R. Watson, Editor. Boca Raton, FL: CRC Press, 1985, 368 pages. ISBN 0-8493-2933-7, $95.00.

An enormous collection of data on virtually every effect, both real and imagined, of nutritional variability on age changes. Included are trace elements, vitamins, fiber, alcohol, exercise, prevention of constipation, nutrition programs, water, and obesity. As is characteristic of multi-authored books, unevenness abounds. The 38 authors have produced chapters as short as three pages and one sufficiently comprehensive to include nearly 400 references. There is little effort to critically interpret the vast amount of data that is presented. Frequently, contradictory conclusions are reached by different authors. There is here a feast of data but with questionable nutritional value and serious risks of mental constipation.

Clinics in Geriatric Medicine—The Aging Process

M.C. Geokas, Guest Editor. Philadelphia: W.B. Saunders Co., 1985, 309 pages. ISSN 0749-0690, $15.00

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This is the first of four books to be published annually in the series "Clinics in Geriatric Medicine." Another attempt at head to toe coverage of age changes. Strehler gets things started with a summary of the field from several perspectives. Hayflick summarizes the Cell Biology of Aging but adds little that is new to the subject. Walford presents his views on caloric restriction and its extension of life span. He takes on the critics of his position with praiseworthy demands for data and straight thinking. One of the more stimulating chapters. There follows a parade of contributions on the aging brain, skeleton, heart, respiratory system, gastrointestinal tract, liver, pancreas, kidney, endocrine system, skin, and, for good measure, aging and atherosclerosis. The cover page says that this is a "Symposium on The Aging Process." Webster's New Collegiate Dictionary says "symposium," which comes from the Greek, has as it's first meaning, ". . . a drinking together, usually following the banquet proper, with music, singing, and conversation; hence, a banquet or social gathering at which there is free interchange of ideas." The contributors to this book never gathered at a symposium, to say nothing of a banquet. Who, then, has been doing the drinking?

Invertebrate Models in Aging Research

D.H. Mitchell and T.E. Johnson, Editors. Boca Raton, FL: CRC Press, 1984, 208 pages. ISBN 0-8493-5823-X, $69.00

How do the optical glands of the *Octopus* influence its lifespan? How is programmed senescence expressed in the coelenterate *Campanularia flexuosa*? These and other fascinating excursions into invertebrate aging are covered in this volume. Other topics in-

cluded are: cell specific gene expression in the roundworm, age-dependent changes in catacholamine-activated adenylate cyclase in nematodes, and progesterone metabolism in aging. *Caenorhabditis elegans. Mercenaria mercenaria*, a clam with a 5 day life span, is introduced. Coverage also includes protozoan aging and neuronal aging in marine mussels and cephalopods. The universality of aging continues to impress, yet most modern textbooks in biology fail even to mention this fundamental property of all living things.

Skin and Aging Processes

B.A. Gilchrest. Boca Raton, FL: CRC Press, 1984, 136 pages. ISBN 0-8493-5472-2, $55.00

Perhaps the first text published entirely devoted to the aging of skin. Included are theories of aging, age-associated changes in normal skin, pathologic processes in aging skin, premature aging syndromes affecting skin, skin cancer, in vitro studies of aging skin, and sun-induced aging. How many of you knew that this is called dermatoheliosis? This is a well-written book by a pioneer in the field of skin aging in vitro. It should enjoy far more circulation than its cost of 40 cents per page will allow.

Physiology of Cell Aging. Interdisciplinary Topics in Gerontology, Volume 18

V.V. Frolkis, Editor, Translated by A. Lipinsky. Basel: Karger, 1984, 206 pages. $89.25.

It is a pleasure to read about the studies done by my countrymen over the last decade at the Institute of Gerontology in Kiev, U.S.S.R. However, I would have preferred to have read it in my native Russian. This institute is the major gerontology institute in the U.S.S.R. and is roughly comparable to the National Institute on Aging in the U.S.A. It is descendent from the famous Kiev Institute of Experimental Biology and Pathology founded and directed by my kinsman Dr. Alexander A. Bogomolets in 1930. I am disappointed to find that his discovery of anti-reticulocyte cytotoxic serum, and its beneficial effects on the aging process, has gone unnoticed in this book. Vladimir Frolkis is the head of the Laboratory of Physiology where he has conducted extensive studies on the nervous systems of aging laboratory animals. Of the ten chapters in the book he is senior author of seven. Topics of study that are reported upon include age changes in the control of aldosterone and in hypothalamic influences on the adrenal cortex, immunological changes with age, and parabiosis and spleen cell grafting between CBA mice of different ages. This book is a useful summary of some of the work done in aging at the most prestigious center for gerontology in the Soviet Union.

The Duke Longitudinal Studies of Normal Aging 1955–1980. An Overview of History, Design, and Findings

E.W. Busse and G.L. Maddox, Editors. New York: Springer, 1985, 192 pages. ISBN 0-8261-4150-1, $26.95.

This monograph provides an introduction and overview of the research results on the multivariate process of aging conducted over the past twenty years in one of the most important longitudinal studies on aging in the world. Highlights cover biological, social, and behavioral aspects of "normal" aging. (Will someone out there please do a book on "abnormal" aging.) Emphasis here is on the organization and coordination of personnel and resources for the conduct of a longitudinal study. This book will be essential reading for all those who are either planning their own 25 year-long longitudinal study of human aging or want to know how this one was conducted.

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